Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3

Elizabeth J. Pishko, Girish S. Kirimanjeswara, Mylisa R. Pilione, Lakshmi Gopinathan, Mary J. Kennett and Eric T. Harvill

Department of Veterinary Science, The Pennsylvania State University, University Park, USA

To assess the contribution of complement to respiratory immunity in the context of a natural bacterial infection, we used mice genetically deficient in complement components and the murine pathogen *Bordetella bronchiseptica*. Complement component C3 was not required for the control of bacterial infection or for the generation of infection-induced protective immunity. However, C3-deficient ($C3^{-/-}$) mice were severely defective, compared to wild type, in vaccine-induced protective immunity. Adoptively transferred immune serum from convalescent wild-type or $C3^{-/-}$ animals rapidly cleared *B. bronchiseptica* from the lungs of wild-type mice but did not affect their growth in $C3^{-/-}$ mice, indicating that the defect is not in the generation of protective immunity, but in its function. Immune serum was effective in C5-deficient mice but had little effect in the lungs of mice lacking either Fcγ receptors (FcγR) or CR3, suggesting bacterial clearance is not via direct complement-mediated lysis. Together, these data indicate that complement is required for antibody-mediated clearance of *Bordetella* and suggest the mechanism involves C3 opsonization of bacteria for phagocytosis that is both CR3- and FcγR-dependent.

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1 Introduction

The complement system is a critical aspect of innate immunity that also contributes to the generation of adaptive immunity [1–3]. Bacterial pathogens can initiate the complement cascade through several major activation pathways leading to cleavage of C3, which generates C3a, a powerful anaphylatoxin involved in signaling and inflammatory cell recruitment, and the opsonin C3b [4, 5]. C3b is also a primary component of the alternative pathway C3 convertase and both C5 convertases. Complement concentrations are relatively low in healthy respiratory tract secretions but can increase rapidly in response to infection [6]. Recent work with C3-deficient ($C3^{-/-}$) mice has revealed important roles for complement within the respiratory tract [1, 7–13]. $C3^{-/-}$ mice exhibit decreased airway hyper-responsiveness and lung eosinophilia in an allergic asthma model [14] and are more susceptible to influenza virus [11]. However, no such role has been established for complement in respiratory immunity to bacterial pathogens. Indeed, although *Mycobacterium avium* is sensitive to complement *in vitro*, $C3^{-/-}$ mice showed no defect in their resistance to this organism [13].

The genus *Bordetella* comprises a group of closely related gram-negative bacterial respiratory pathogens with a broad host range. *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough in humans while *B. bronchiseptica* infects a number of non-human mammals, causing atrophic rhinitis in pigs, snuffles in rabbits and kennel cough in dogs. *B. pertussis* and *B. parapertussis* have recently been reclassified as subspecies of *B. bronchiseptica*, due to their extremely close relatedness [15]. The two human pathogens appear to have made recent independent host range jumps from a *B. bronchiseptica*-like progenitor to emerge as pathogens that efficiently infect and spread amongst humans [15]. However, both *B. pertussis* and *B. parapertussis* are more closely related to *B. bronchiseptica* than they are to each other [15]. Since *B. bronchiseptica* is genetically manipulatable and naturally infects mice, it provides a powerful model system to examine the molecular interactions between a bacterial

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Abbreviations: LRT: Lower respiratory tract **i.n.:** Intranasal **MAC:** Membrane attack complex

pathogen and the host immune response within the respiratory tract.

We have shown that antibodies are necessary and sufficient to clear *B. bronchiseptica* from the lower respiratory tract (LRT) of mice [16], and *B. bronchiseptica* has been shown to activate compliment *in vitro* [17]. Based on the known roles of complement in innate immunity as well as in the generation and function of adaptive immunity, we hypothesized that complement would be required for some aspect of the *in vivo* immune response to *B. bronchiseptica*. Interestingly, $C3^{-/-}$ mice showed no defect in their ability to control and clear *B. bronchiseptica* from the respiratory tract and generated effective protective immunity that rapidly cleared a subsequent infection. However, vaccination of $C3^{-/-}$ mice with heat-killed *B. bronchiseptica*, while resulting in detectable antibody titers, did not confer any protection against bacterial challenge.

Vaccinated $C3^{-/-}$ mice generate antibodies that are effective in rapidly clearing *B. bronchiseptica* from wild-type mice. However, immune serum from either wild-type or $C3^{-/-}$ mice had no effect on bacterial numbers in $C3^{-/-}$ mice. Immune serum rapidly cleared *B. bronchiseptica* from C5-deficient ($C5^{-/-}$) mice but was less effective in FcγR-deficient (Fc$\gamma R^{-/-}$) or $CR3^{-/-}$ mice, indicating that the primary function of antibody-mediated complement activation is not the direct lysis of bacteria but facilitation of their removal by phagocytic cells. Together these data indicate that complement can contribute to antibody-mediated clearance of bacterial pathogens within the respiratory tract, and reveal a potentially important mechanistic difference between vaccine-induced and infection-induced immunity.

2 Results

2.1 Normal immune responses of C3-deficient mice to *B. bronchiseptica* infection

In order to investigate the contribution of complement to the immune response of mice during an initial *B. bronchiseptica* infection, we compared the ability of wild-type C57BL/6 and congenic $C3^{-/-}$ mice to clear bacterial infection from three regions of the respiratory tract. Mice were inoculated with 5×10^5 CFU in a volume of 50 μl PBS applied intranasally (i.n.), a method shown to distribute bacteria throughout the respiratory tract [18]. On days 0, 7, 14, 28, 49, 70 and 105 post-inoculation bacterial colonization levels were determined in the nasal cavity, trachea and lungs. No significant difference was observed in the levels of *B. bronchiseptica* colonization between wild-type and $C3^{-/-}$ mice on any of the indicated days post-inoculation (Fig. 1), indicating that C3 was not required for effective clearance of *B. bronchiseptica* from the respiratory tracts of mice.

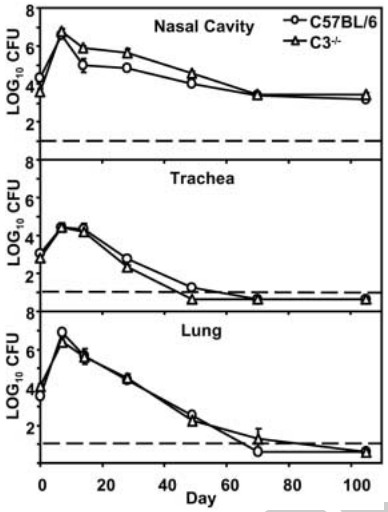

Fig. 1. Clearance of *B. bronchiseptica* infection from the respiratory tract. Groups of four 4–6-week-old C57BL/6 and $C3^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS to the external nares. Bacterial numbers recovered from the nasal cavity, trachea, and lungs at the indicated times post-inoculation are expressed as the $\log_{10}$ mean/organ ± SE. Data are representative of multiple experiments. Dashed line represents the lower limit of detection for the assay.

To determine whether complement is required for the control of a secondary infection with *B. bronchiseptica* after a primary infection has been cleared from the LRT, C57BL/6 and $C3^{-/-}$ mice were inoculated with 5×0^5 CFU in a volume of 50 μl, as described above, and allowed to clear the LRT. These mice were then challenged with a second inoculum of *B. bronchiseptica* on day 70 post-inoculation, as described above, and bacterial numbers in the nasal cavity, trachea and lungs were determined 3 days after the second inoculation. In both mouse strains bacterial numbers were only a fraction of those found 3 days after primary infection (Fig. 1); approximately 1/100 in the nasal cavity, 1/1,000 in the trachea and 1/3,500 in the lungs (Fig. 2). Since no significant difference was observed between wild-type and $C3^{-/-}$ strains, mice were apparently capable of mounting an efficient anamnestic response to *B. bronchiseptica* in the absence of a functional complement cascade.

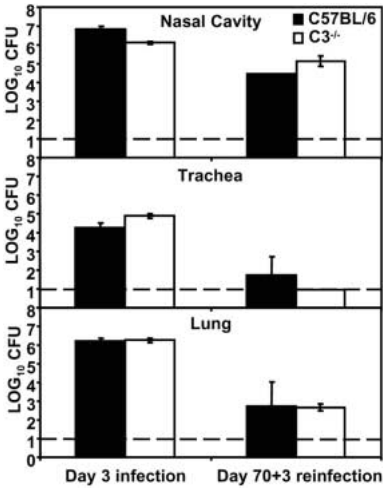

Fig. 2. Clearance of *B. bronchiseptica* from the respiratory tract after an anamnestic response. Groups of four 4–6-week-old C57BL/6 and $C3^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS to the external nares. One group of each mouse strain was sacrificed on day 3 and another allowed to clear the LRT. On day 70 post-inoculation a second 5×10^5 CFU of *B. bronchiseptica* was delivered to the nares as described above. Bacterial numbers recovered from the nasal cavity, trachea, and lungs on day 3 after initial infection or day 3 after the second inoculation, depending on treatment group, are expressed as the $\log_{10}$ mean/organ ± SE. Data are representative of multiple experiments. Dashed line represents the lower limit of detection for the assay.

2.2 Vaccine-mediated clearance of *B. bronchiseptica* from the lung

Although substantial differences have been noted between the protective immunity generated in response to infection and vaccination, there is little understanding of the mechanisms involved in these differences. In both clinical studies and mouse experiments infection-induced immunity has been shown to be more protective than vaccination-induced immunity ([19, 20] and unpublished data). To examine whether differences in the involvement of complement could be responsible for these observations, we examined the role of C3 in effective vaccine-induced protection against *B. bronchiseptica*. Groups of C57BL/6 and $C3^{-/-}$ mice were vaccinated i.p. with 10^8 CFU heat-killed *B. bronchiseptica*, followed by a second dose 2 weeks later. Two weeks after the second vaccination, the mice were challenged i.n. with 5×10^5 CFU of *B. bronchiseptica* as described above. Animals were sacrificed on day 3 post-challenge and respiratory tract colonization levels were determined as described above.

As previously shown, vaccination had little effect on bacterial numbers in the nasal cavity and a moderate effect in the trachea that was not complement-dependent (Fig. 3A). In the lungs of wild-type animals, vaccination reduced bacterial numbers to 1/60,000 that of naive animals on day 3 post-inoculation. However, vaccinated $C3^{-/-}$ mice exhibited only a 30-fold reduction in CFU in the lungs and had more than 2,000 times the number of bacteria present in the lungs of vaccinated wild-type mice (Fig. 3A). Since complement can contribute to the generation of adaptive immunity, we compared the anti-*B. bronchiseptica* titers of various antibody isotypes in the serum of vaccinated wild-type and $C3^{-/-}$ mice. Although $C3^{-/-}$ mice had IgM titers similar to that of wild-type mice, their titers of each IgG isotype were substantially lower (Fig. 3B).

2.3 Role of complement in antibody-mediated clearance of *B. bronchiseptica* from the LRT

Since complement is involved in both the generation and function of antibodies, the failure of vaccinated $C3^{-/-}$ mice to clear *B. bronchiseptica* could be due to a defect in either antibody generation or function. To discriminate between these possibilities we performed reciprocal adoptive transfer of immune serum between wild-type and $C3^{-/-}$ mice followed by challenge with *B. bronchiseptica*. Heat-inactivated serum (usually 0.2 ml) from wild-type or $C3^{-/-}$ mice that were naive (naive serum) or convalescent from *B. bronchiseptica* infection 28 days post-inoculation (convalescent serum) was adoptively transferred i.p. into wild-type or $C3^{-/-}$ mice. We then immediately challenged these mice with 5×10^5 CFU of *B. bronchiseptica* i.n. as described above.

Naive serum had no effect on bacterial numbers in either mouse strain. Wild-type convalescent serum rapidly cleared *B. bronchiseptica* from the LRT of wild-type mice from day 3 post-inoculation/transfer onward, but had no effect on $C3^{-/-}$ mice (Fig. 4). As little as 0.02 ml of immune serum was able to lower bacterial numbers significantly, with 0.1 ml and higher able to reduce bacterial numbers in wild-type mice to 1/1,000 that of untreated wild-type mice on day 3 post-inoculation/transfer, whereas up to 1 ml of that same serum had no effect on bacterial numbers in $C3^{-/-}$ mice (data not shown). Convalescent serum from $C3^{-/-}$ mice was as effective as serum from wild-type mice in clearing *B. bronchiseptica* from the LRT of wild-type mice (data not shown). Similarly, $C3^{-/-}$ vaccine serum was effective in clearing *B. bronchiseptica* from the LRT of wild-type mice, indicating that functional impairment of serum antibodies rather than their generation is responsible for the defect observed in vaccinated $C3^{-/-}$ mice. Therefore, independent of any

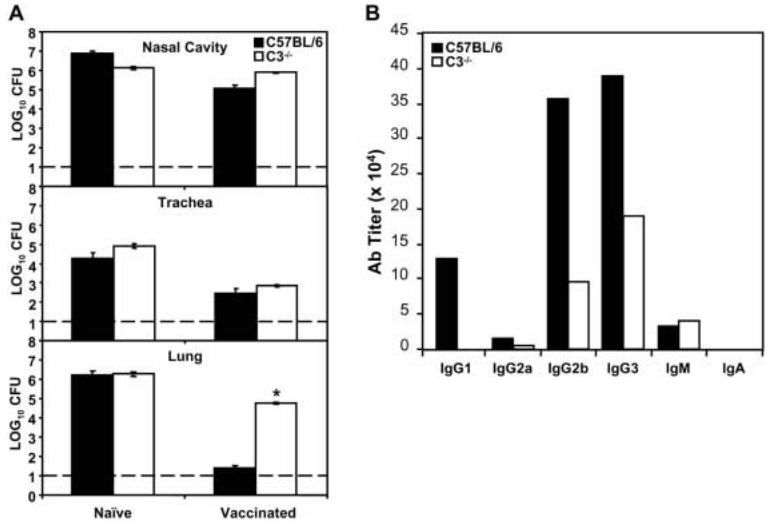

Fig. 3. Vaccine-mediated clearance of *B. bronchiseptica* from the lung. (A) Groups of four 4–6-week-old C57BL/6 and $C3^{-/-}$ mice were immunized i.p. twice at 2-week intervals with 10^8 CFU of heat-killed *B. bronchiseptica*. Two weeks after the second vaccination, animals were challenged i.n. with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS. Bacterial numbers recovered from the nasal cavity, trachea, and lungs on day 3 post-challenge are expressed as the $\log_{10}$ mean/organ ± SE. Data are representative of multiple experiments. Dashed line represents lower limit of detection for the assay. *Significantly different from vaccinated wild-type animals ($p<0.0001$). (B) Antibody titers determined by ELISA on pooled serum taken from vaccinated C57BL/6 and $C3^{-/-}$ mice on day 28 post-inoculation with 5×10^5 CFU of *B. bronchiseptica* delivered i.n. in a 50-μl volume of PBS. Samples were run in triplicate.

effect on antibody generation, $C3^{-/-}$ mice are severely defective in antibody functions involved in mediating bacterial clearance.

There are several ways in which complement could be acting in conjunction with antibodies to clear bacterial infections in the lung. Complement may directly lyse bacteria via formation of the membrane attack complex (MAC), it may attract and/or activate phagocytic cells, and it may facilitate phagocytosis and bacterial killing by phagocytic cells. In order to determine whether direct complement-mediated lysis was occurring, we adoptively transferred serum into wild-type and $C5^{-/-}$ mice as described above. Antibodies effectively reduced the numbers of *B. bronchiseptica* in the tracheas and lungs of both strains of mice on day 3 post-inoculation (Fig. 5). Since $C5^{-/-}$ mice lack C5b, a critical part of the MAC, and these mice reduced bacterial numbers to the same extent as wild-type mice after adoptive transfer of antibodies, it appears that direct MAC-mediated bacterial lysis is not required for bacterial clearance. $Fc\gamma R^{-/-}$ mice, which are defective in FcγR-mediated phagocytic functions but have a normal complement system, failed to clear *B. bronchiseptica* from the lungs after adoptive transfer of antibodies (data not shown). Together these data suggest that antibody-mediated bacterial clearance requires complement not to directly lyse bacteria, but to facilitate phagocytosis via FcγR.

2.4 Recruitment of inflammatory cells to the lungs during *B. bronchiseptica* infection

Activation of the complement cascade results in the release of numerous factors that can attract and activate phagocytic cells. Although effective antibody-mediated clearance of *B. bronchiseptica* from $C5^{-/-}$ mice indicates that C5a is not required, other factors, including C3a, are not dependent on C5 and may affect recruitment and activation of phagocytes. We therefore examined inflammatory cell recruitment in the presence and absence of C3. Lungs were excised from *B. bronchiseptica*-infected wild-type and $C3^{-/-}$ mice that had been adoptively transferred naive or convalescent serum, as described above. Formalin-fixed, and hematoxylin and eosin (H&E)-stained sections were examined by a trained mouse pathologist blinded as to the treatment.

On day 1 post-transfer and -inoculation there was no detectable difference in cell recruitment or tissue damage between the wild-type or $C3^{-/-}$ mice regardless of the serum used in the transfer (Fig. 6A). Lesions included perivascular and peribronchiolar lymphoid cuffing, suppurative alveolar pneumonia, and mixed inflammatory cell infiltrates. Lungs from wild-type mice given naive serum, and $C3^{-/-}$ mice given naive, or immune serum, exhibited similar lesions and inflammatory cell infiltrates on day 3 post-transfer and -inoculation, and contained

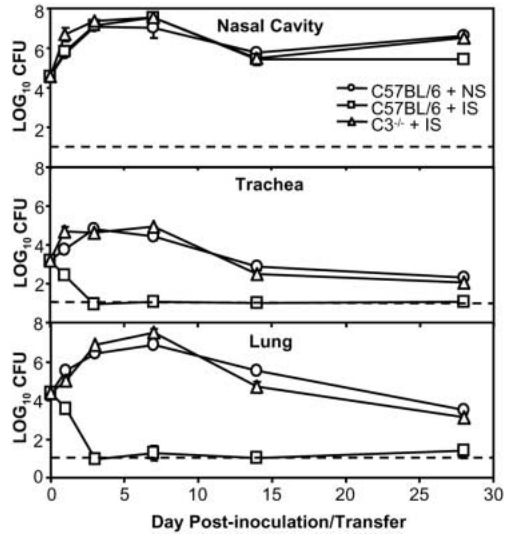

Fig. 4. Adoptive transfer of serum antibodies to C3$^{-/-}$ mice. Groups of four 4–6-week-old C57BL/6 and C3$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS to the external nares. Two-hundred microliters of naive serum (NS) or convalescent serum (IS) was given by i.p. injection prior to inoculation. Bacterial numbers recovered from the nasal cavity, trachea, and lungs on the indicated days post-inoculation are expressed as the $\log_{10}$ mean/organ ± SE. Data are representative of multiple experiments. Dashed line represents the lower limit of detection for the assay.

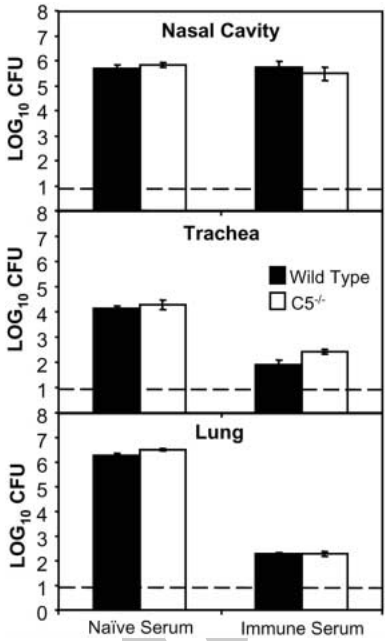

Fig. 5. Adoptive transfer of serum antibodies to C5$^{-/-}$ mice. Groups of four 4–6-week-old B10.D2-H2^{d}H2-T18^{c}Hc^1/nSnJ (wild-type) and B10.D2-H2^{d}H2-T18^{c}Hc^0/nSnJ (C5$^{-/-}$) mice were inoculated with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS to the external nares. Two-hundred microliters of naive serum or convalescent serum (immune serum) was given by i.p. injection prior to inoculation. Bacterial numbers recovered from the nasal cavity, trachea, and lungs on day 3 post-inoculation are expressed as the $\log_{10}$ mean/organ ± SE. Dashed line represents the lower limit of detection for the assay.

similar high numbers of bacteria (Fig. 4). Wild-type mice given immune serum had the highest overall lesion scores on day 3, although they had cleared bacteria by this time (Fig. 6A). C5$^{-/-}$ mice showed no significant difference in histopathology on day 3 post-inoculation and -adoptive transfer when compared to wild-type controls treated similarly (data not shown). The lack of either C3a or C5a appears to have little if any effect on pathological lung inflammation.

In order to quantify inflammatory cell trafficking to the lungs in response to *B. bronchiseptica* infection in the presence and absence of C3, we determined the number of total leukocytes, macrophages, neutrophils and lymphocytes in the lungs 12 h post-inoculation. No significant differences were observed in the total number of cells (Fig. 6B) or in any of the individual cell subpopulations (Fig. 6C) in the lungs between wild-type and C3$^{-/-}$ mice, suggesting mice can mount a normal inflammatory response to *B. bronchiseptica* in the absence of complement. Since antibodies can accentuate the inflammatory response by activating the classical complement pathway, we investigated inflammatory cell recruitment to the lungs in the presence of adoptively transferred antibodies in wild-type and C3$^{-/-}$ mice. Serum antibodies significantly increased the numbers of total cells and neutrophils recovered from both wild-type and C3$^{-/-}$ mice (Fig. 6B, C), indicating that C3 is not essential for cell recruitment to this organ during a *B. bronchiseptica* bacterial infection.

2.5 Role of CR3 in antibody-mediated clearance of *B. bronchiseptica* from the LRT

To distinguish between the effects of C3a on recruitment and activation of phagocytes from the effects of C3b opsonization on phagocytosis, we used mice lacking the α subunit (CD11b) of CR3, the primary receptor involved in recognizing and phagocytosing iC3b-coated bacteria. CR3$^{-/-}$ mice were adoptively transferred naive or convalescent serum just prior to inoculation with 5×10^5 CFU of *B. bronchiseptica* as described above. CR3$^{-/-}$ mice treated with immune serum were substantially deficient in their ability to clear *B. bronchiseptica* from the LRT on day 3 post-inoculation compared to wild-type mice

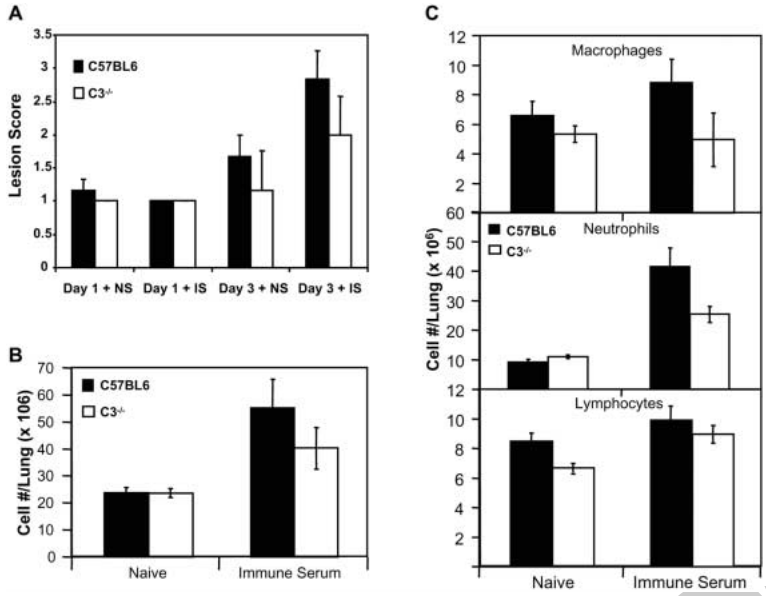

Fig. 6. Cell recruitment in the lungs of wild-type and $C3^{-/-}$ mice adoptively transferred with serum antibodies. (A) For histological evaluation, lungs were taken for fixing and H&E staining from both C57BL/6 and $C3^{-/-}$ mice infected with *B. bronchiseptica* on days 1 and 3 post-adoptive transfer of either naive (NS) or convalescent serum (IS). Data are expressed as the mean lesion score ± SE of the stained lung sample slides. (B) For total leukocyte cell recruitment, lungs from three separate animals were digested and leukocytes separated by density gradient centrifugation. Cells were enumerated visually on a hemacytometer and are reported as the total number of cells per lung. (C) Cell differentials were determined after isolated leukocytes were spun onto glass slides and Wright-Giemsa stained. Total number of each cell type was determined by counting random microscope fields. Cell numbers are reported as total cells of that type per lung ± SE, based on percentage of each type seen on slides.

(Fig. 7). Colonization levels were reduced to approximately 1/6 and 1/500 the untreated levels in the trachea and lung of $CR3^{-/-}$ mice, respectively. In contrast, antibody treatment reduced bacterial numbers to 1/4,000 and 1/100,000 of untreated levels in wild-type mice. Although there was a small CR3-independent antibody effect, CR3 appears to be required for serum antibodies to fully and efficiently clear *B. bronchiseptica* from the LRT of mice.

3 Discussion

Even in relatively simple *in vitro* experiments, the numerous serum proteins that comprise the complement system are known to contribute a complex set of signals and activities that are affected by the concentrations, proximity and orientation of many activators and inhibitors [2, 21, 22]. In addition, the effects of complement activation are highly dependent on the nature of the target, and bacterial pathogens have evolved multiple mechanisms to evade these effects [23–27]. It would therefore be difficult to extrapolate from even the most rigorous *in vitro* studies to the *in vivo* role of complement in respiratory tract immunity. Conditions that affect such assays are likely to differ dramatically from the basal aqueous sol to the surfactant- and mucus-rich air interface of the respiratory mucosa. Here we examined the role of complement in respiratory immunity by measuring its contribution to the control and clearance of a respiratory pathogen in a natural host infection model. While our initial experiments in $C3^{-/-}$ mice indicate that complement is not required to control *B. bronchiseptica* infection and limit it to the upper respiratory tract, it is certainly required for effective vaccine-induced immunity and for the actions of adoptively transferred antibodies in the LRT.

There are multiple mechanisms by which complement could contribute to the elimination of bacteria from the LRT. The most direct is by MAC formation, which leads to lysis of bacterial cells through membrane disruption. We have previously shown that antibodies very efficiently activate complement to directly lyse *B. bronchiseptica* by this mechanism *in vitro*, even when serum is diluted in PBS to concentrations as low as 1% [17]. However, this mechanism is apparently not sufficient to eliminate *B. bronchiseptica* from the lungs of mice, since antibodies did not clear the bacteria from $Fc\gamma R^{-/-}$ mice, which have an intact complement system, but did clear

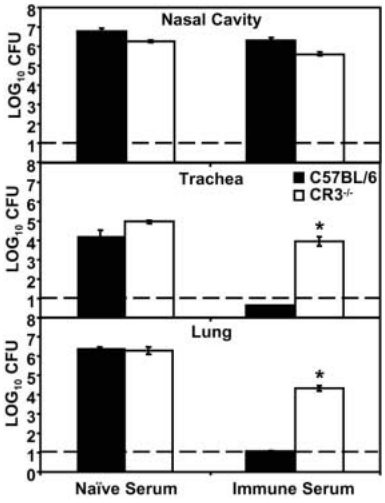

Fig. 7. Adoptive transfer of serum antibodies in CR3$^{-/-}$ mice. Groups of four 4–6-week-old C57BL/6 and CR3$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. bronchiseptica* delivered in a 50-μl volume of PBS to the external nares. Two-hundred microliters of naive serum or convalescent serum (immune serum) was given by i.p. injection prior to inoculation. Bacterial numbers recovered from the nasal cavity, trachea, and lungs at the indicated times post-inoculation are expressed as the $\log_{10}$ mean ± SE. Dashed line represents the lower limit of detection for the assay. *Significantly different from immune serum-treated wild-type animals ($p<0.0001$).

the bacteria from C5$^{-/-}$ mice, which lack the ability to form the MAC.

The other two likely mechanisms of complement-mediated bacterial clearance involve effector cells. Phagocytic cells can be recruited and activated by the diffusible molecules that are released by the catalytic breakdown of complement components. Alternatively, molecules that are deposited on bacterial membranes can act as ligands for receptors, such as CR3, that mediate phagocytosis and killing of bacterial cells [28, 29]. Since antibodies are also efficient opsonins, and the FcR that they bind are required for efficient antibody-mediated clearance, we hypothesized that complement may be acting primarily to recruit and activate FcγR-bearing phagocytes. However, we observed no significant difference between wild-type and C3$^{-/-}$ mice in their recruitment of inflammatory cells (Fig. 6). Furthermore, antibodies were effective in C5$^{-/-}$ mice, where C3b opsonization occurs but the highly active chemoattractant C5a is missing. In addition, antibodies had little effect in CR3$^{-/-}$ mice, indicating that complement-mediated opsonization is required for efficient antibody-mediated clearance. These experiments indicate that C5a is not required for efficient antibody-mediated clearance; and since CR3 is required it is likely that both MAC formation and complement-dependent recruitment are not sufficient for clearance in the absence of complement-mediated opsonization.

Although not the focus of this study, these experiments revealed substantial, and potentially important, differences between infection-induced and vaccination-induced protective immunity. Both vaccination and adoptively transferred serum conferred protective immunity that was dependent on complement. We have also observed that vaccination does not confer protection in B cell-deficient MuMT mice but adoptive transfer of vaccine-induced serum does (unpublished data). Together these data suggest that vaccination induces primarily serum antibodies which require complement to effectively clear *B. bronchiseptica* infection from the LRT. In contrast, infection-induced immunity conferred protection that was independent of complement, suggesting mucosal infection induces some immune mechanism independent of serum antibodies. *B. bronchiseptica* is not believed to invade tissues or cells, and there is no obvious mechanism for clearance by T cells. In addition, MuMT mice do not generate protective immunity [17], indicating that antibodies are required. Since systemic vaccination does not result in efficient generation of mucosal IgA, we propose that infection-induced immunity results in the production of IgA, which clears *B. bronchiseptica* by complement-independent mechanisms. The failure to produce substantial secretory IgA may explain certain shortcoming of vaccines to Bordetellae, as well as to other mucosal pathogens, and suggests strategies to overcome these shortcomings.

Together, these data support a detailed model for antibody-mediated clearance of *B. bronchiseptica* from the LRT of mice. We propose that antibodies activate complement to opsonize bacteria which are then phagocytosed via a combination of both CR3 and FcγR. This is consistent with the phosphatidylinositol 3-kinase-dependent model proposed by Jones et al. for activation of phagocytosis by human neutrophils [29]. In this model, ligation of antibody-bound FcγR on the neutrophil surface trigger activation of CR3 through the phosphatidylinositol 3-kinase pathway, which leads to increased avidity of CR3 for bacterial surface-bound iC3b [29, 30]. We are currently investigating the contributions of individual FcγR as well as the role of CR3 in serum antibody-mediated clearance of the Bordetellae. It will also be important to determine whether this mechanism of clearance is common to vaccine-induced protection from other respiratory pathogens.

4 Materials and methods

4.1 Bacteria

B. bronchiseptica RB50 (wild type) was obtained after a single passage from an original rabbit isolate [31]. Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 7.5% defribrinated sheep blood and 20 μg/ml streptomycin. Bacteria were grown in Stainer-Scholte broth with supplements and 20 μg/ml streptomycin to mid-log phase (optical densities of approximately 0.3 at 600 nm) at 37°C on a roller drum for experiments.

4.2 Mice

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME), and $C3^{-/-}$ mice, back-crossed extensively onto a C57BL/6 background, have been described elsewhere and were kind gifts of Dr. Rick Wetsel [8]. $C5^{-/-}$ mice (B10.D2-$H2^{d}$H2-T18^{c}Hc^{0}/nSnJ) and their parent strain (B10.D2-$H2^{d}$H2-T18^{c}Hc^{1}/nSnJ) were also obtained from Jackson Laboratories. $CR3^{-/-}$ mice (C57BL/6 background) were the kind gift of Dr. Christie M. Ballantyne and have also been previously described [32].

4.3 Inoculation and immunization protocols

Mice were lightly sedated with isoflurane, and inoculated i.n. by pipetting 5×10^{5} CFU of bacteria in 50 μl onto the tip of the external nares. For the time-course experiment groups of four animals were sacrificed on days 0, 3, 5, 7, 14, 28, 49, 70 and 105 post-inoculation. Colonization levels were determined by homogenizing the indicated organ in 1× PBS and plating aliquots for colony counts. The nasal cavity and trachea were homogenized in 500 μl of PBS and lungs in 1 ml PBS. The homogenates and necessary dilutions were plated in 50-μl volumes onto Bordet-Gengou agar with streptomycin. Colonies were counted after 2 days incubation at 37°C. For re-infection, animals were infected as described above and then 70 days post-infection challenged with a second i.n. dose of 5×10^{5} CFU of *B. bronchiseptica*. On day 3 post-challenge mice were killed and colonization levels determined as described above.

For vaccination, animals were immunized i.p. twice at 2-week intervals with 10^{8} CFU of heat-killed *B. bronchiseptica*. Heat-killed bacteria were prepared by incubating bacteria grown to mid-log phase at 80°C for 30 min. At 2 weeks after administration of the second vaccination, animals were challenged i.n. as described above and killed on day 3 post-challenge for determination of colonization levels as described. For passive transfer of antibodies, wild-type mice were inoculated as described and serum was collected on day 28 post-inoculation. Titers of anti-*B. bronchiseptica* antibodies in the convalescent sera were determined by ELISA as previously described [33]. Injection of 200 μl of this convalescent serum i.p. into naive mice was immediately followed by i.n. inoculation as described above. Animals were killed on the indicated day post-transfer and inoculation and colonization determined as described. Animals were handled in accordance with institutional guidelines, in keeping with full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International.

4.4 Histological evaluation

The lungs from treated mice were removed, inflated and fixed with 10% neutral-buffered formalin, embedded in paraffin, and sectioned. Slides were routinely prepared and stained with H&E. An assessment of microscopic lesions was made by one of the authors (M.J.K.), experienced in rodent pathology and blinded to experimental treatment. Descriptive evaluations of the lesions were recorded, and lung lesions were graded using a scale of 0 to 4. Sections with no lesions and no inflammation were given a score of 0, a score of 1 indicated slight inflammation with few or scattered lesions and <10% of lung fields affected, 2 indicated mild lesions with 10–20% of lung fields affected, 3 indicated moderate lesions with 20–30% of the lung fields affected, and those given grade 4 were characterized by extensive lesions, marked inflammation, and >30% of the lung affected.

4.5 Preparation of lung leukocytes

Lung leukocytes were isolated as previously described [34]. Briefly, the lungs were perfused with 2 ml of PBS, excised, rinsed in PBS, minced, and enzymatically digested for 2 h at 37°C using 3 ml of digestion buffer consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1% penicillin-streptomycin solution (10,000 μg/ml), 1 mg/ml collagenase and 10 μg/ml DNase. Lung leukocytes were isolated using density gradient centrifugation and then counted on a hemacytometer. Cell differentials (neutrophils, macrophages, and lymphocytes) were visually counted from Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides (Shandon Cytospin, Pittsburgh, PA). A total of 300 cells were counted from randomly chosen high-powered-microscope fields for each sample. The absolute number of a leukocyte subset was calculated by multiplying the percentage of each subset in an individual sample by the total number of lung leukocytes in that mouse. Statistical significance of data points was determined using a Student's unpaired *t*-test.

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Correspondence: Eric T. Harvill, The Pennsylvania State University, Department of Veterinary Science, 115 Henning Bldg., University Park, PA 16802, USA
Fax: +1-814-863-6140
e-mail: harvill@jjpsu.edu

