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DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

MURINE TRANSMISSION AND A POTENTIAL ENVIRONMENTAL RESERVOIR OF *BORDETELLA BRONCHISEPTICA*

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Abstract

Bordetella pertussis, the causative agent of whooping cough, infects and sickens millions around the world every year, both in wealthy and resource-constrained regions. In order to protect human populations, we need to move beyond the current vaccine and treatment methods and gain a greater understanding of how it transmits. *Bordetella bronchiseptica*, a related pathogen which primarily affects non-human mammals, is used in laboratory settings to study these transmission dynamics. To understand how the immune system functions in controlling transmission, we infected immune-compromised mice with *B. bronchiseptica* and observed the importance of TLR4-induced innate immunity in controlling colonization, shedding, and transmission, and the correlation of the neutrophil response to higher shedding of infectious particles. We also studied the intracellular survival of *B. bronchiseptica* in *Dictyostelium discoideum*, a common soil amoeba, and showed that non-mammalian eukaryotic cells are a potential environmental reservoir. By understanding how *B. bronchiseptica* transmits through investigation of relevant immune mechanisms and environmental reservoirs, we can move closer to finding ways to interrupting the chain of transmission of *B. pertussis* in humans.

Table of Contents

List of Figures

Acknowledgments

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Introduction

The bacterial genus *Bordetella* is most widely known for containing the species *Bordetella pertussis*, the causative agent of whooping cough. It is a gram-negative bacillus first discovered in 1906 by Jules Jean Baptiste Vincent Bordet and Octave Gengou. Around that time, it was the leading infectious disease contributing to death in children (Weisberg, 2007). One of the most recent estimates of the disease burden by the WHO stated that, in 2008, there were about 16 million cases of the disease resulting in just under 200,000 deaths, almost entirely in developing countries (World Health Organization, 2013). For those who come down with the disease, the direct and indirect costs can be substantial (Caro, et al., 2005)

B. pertussis infects the trachea and bronchi and is most severe in young children who may have not yet completed their vaccination schedule, although recently, incidence in adults is increasing due to waning immunity (Steele, 2004; Pluta, Lynm, & Glass, 2010). The cough can be severe enough to cause fractured ribs. It often lasts months, although many patients ultimately recover (Pluta, Lynm, & Glass, 2010). The repeated coughs often force the afflicted person to make a "whoop" sound as they try to inhale. Other side effects of the coughing can include vomiting, hernias, seizures, lack of oxygen, and bleeding in the eyes (Weisberg, 2007). It is also common for people to carry the bacteria without displaying the obvious, most severe symptoms of whooping cough, allowing them to become carriers of the disease (Weisberg, 2007). In fact, it is estimated that about 13% of all coughing illnesses in the US are attributable to *B. pertussis* (Cherry, 2003). The number of cases in developed countries appears to be on the rise; this may, however, be due to increased surveillance and better testing methods (Cherry, 2003).

A vaccine does exist for *B. pertussis*. The current schedule of three primary doses and two boosters is plausible to complete in developed countries, but difficult to implement in the under-resourced areas where the burden is the worst. Additionally, the vaccine is only about 80% effective, far less than many other vaccine-preventable diseases (Steele, 2004). After the last booster at age 6, protection begins to decrease between ages 11 and 15, and the fact that many adults do not get boosters lead them to be potential reservoirs through which *B. pertussis* can be passed to younger, more vulnerable, populations (Steele, 2004).

Vaccines are generally the go-to method of controlling infectious diseases. For example, in the global fight against HIV, one of the primary focuses has been to develop a vaccine (Koff, 2010). Until this vaccine is developed, the focus of anti-HIV efforts have focused on public health measures, such as encouraging the use of condoms, based on knowledge of the transmission dynamics of HIV (Ratzan, 2011). Although millions have been saved by the vaccine against *B. pertussis*, it is still not as effective as it needs to be, the dosage schedule is complicated, and immunity wanes over time without boosters. More research needs to be done on the transmission dynamics of *B. pertussis* to try to unveil potential methods of interrupting the chain of transmission other than via the vaccine, just as is currently being done with HIV.

To understand the control of transmission of infectious diseases, it is important to understand how host immunity functions to control the progression and transmission of disease. Host transmission can determine the tendency of an individual to catch and develop an infection and also how infectious it will then be to others (Pathak, Creppage, Werner, & Cattadori, 2010). Innate immunity is one of two major parts of the immune system, in which pattern-recognition receptors (PRRs) have evolved to recognize molecules called pathogen-associated molecular patterns (PAMPs), which are typically associated with a variety of pathogens. A major class of

PRRs are the Toll-like receptors (TLRs), of which TLR4 is particular important in controlling *Bordetella* infections since it recognizes lipopolysaccharide (LPS), an outer membrane molecule characteristic of Gram-negative bacteria such as *Bordetella* (Kawai & Akira, 2010). *Bordetella bronchiseptica*, a species related to *B. pertussis*, causes respiratory disease in a variety of animals and is used for modeling *Bordetella* infections in mice; it was used in one study to show the necessity of TLR4 in mounting a response to infection. Mice lacking TLR4 could not mount a satisfactory cytokine response to combat the infection, which caused fatal pneumonia in many cases (Mann, Kennett, & Harvill, 2004).

The other major part of the immune system is adaptive immunity. Whereas the receptors of the innate system are determined at birth by DNA, the receptors of the B and T cells of the adaptive immune system can develop to recognize unique antigens and have "memory", which means that they react efficiently to presentation of previously seen pathogens. T cells mature in the thymus from precursors originating in the bone marrow, and have many different functions. The most prevalent T cell type, the CD4+ "helper" T cell, releases cytokines and sends cellular signals by direct contact to assist other cells in clearing infections. B cells, which develop in the bone marrow, are the producers of antibodies, which are proteins that help to clear infections by neutralizing pathogens or marking them for destruction (Bonilla & Oettgen, 2010). Thus far, no studies have suggested how T and B cells might function in controlling transmission of *Bordetella*.

Aside from the direct host-to-host transmission via airborne droplets from coughing, the potential for an environmental reservoir of *Bordetella* has become apparent. The discovery that *Bordetella* is able to survive intracellularly in human macrophages by avoiding destruction after phagocytosis (Lamberti, Hayes, Perez Vidakovics, Harvill, & Rodriguez, 2010) suggests that

perhaps other eukaryotic cells in the environment could also be infected, providing an intermediate step for transmission between hosts. For example, the common soil amoeba *Dictyostelium discoideum* is often used in experiments on intracellular survival of certain pathogens since it is able to be infected by many different bacteria such as *Pseudomonas, Legionella, and Mycobacteria* (Lima, Lelong, & Cosson, 2011). The survival of bacteria within environmental amoeba has the potential to protect them from disinfectants, or other environmental stresses, which may otherwise kill them outside the host (Caeiro, et al., 2012). Especially in hospitals, amoeba have been shown to assist in the transmission of infections such as of *Legionella pneumophila* (Caeiro, et al., 2012). The use of amoeba to study virulence of intracellular microbes has raised the concern that the same virulent traits that allow them to infect the amoeba also allow them to infect their normal mammalian hosts, selecting for the more dangerous bacteria to survive in the reservoir (Caeiro, et al., 2012).

Bordetella species have the ability to exist in one of several phases, which determine their ability to cause disease or focus on survival in a less-than-ideal environment. The *bvgAS* locus regulates the switch between the Bvg+ and Bvg- phases (Cotter & Miller, 1994). It does this upon signals transmitted from the BvgS sensor protein/BvgA regulatory protein in response to environmental triggers, such as temperature variations and certain chemicals, such as nicotinic acid (Beier, Schwarz, Fuchs, & Gross, 1995). The Bvg+ phase results in production of virulence factors like toxins, such as adenylate cyclase-hemolysis and dermonecrotic toxin, and adhesins, such as fimbriae and pertactin. The Bvg- phase can result in motility through production of flagella, and is hypothesized to be more suited for survival in an environmental reservoir; *Bordetella* switch to Bvg- when in a non-ideal environment, such as in the presence of nicotinic acid or low temperature (Cotter & Miller, 1994).

In this thesis, we aim to investigate transmission dynamics of *B. pertussis* in order to better understand how to interrupt the chain of transmission and prevent the millions of cases of illness attributed to it each year. We infected mice with *B. bronchiseptica* in order to investigate the roles of innate and adaptive immunity in controlling transmission and showed the importance of TLR4-mediated innate immunity in preventing shedding, colonization, and therefore transmission. Additionally, we observed that the neutrophil response to infection correlates to increased infectiousness. Finally, we found that *D. discoideum* is a potential environmental reservoir for *Bordetella* species by observing that *B. bronchiseptica* can survive intracellularly in the amoeba.

Materials and Methods

Bacterial cultures

We used several strains of *B. bronchiseptica*. RB50, referred to hereafter as "wild-type", was a *B. bronchiseptica* strain isolated from a New Zealand White rabbit. RB53 is the RB50 mutant phase-locked Bvg+, and RB54 is the RB50 mutant phase-locked Bvg- (Cotter & Miller, 1994). All *Bordetella* were grown in Stainer-Scholte media (Stainer & Scholte, 1971) and the *Klebsiella aerogenes* was grown in Luria-Bertani (LB) broth at 37°C to mid-log phase growth (optical density .500-1.000 at 600nm) before being used in experiments. *Bordetella* was grown on plates of Bordet-Gengou (BG) (Difco) agar with 10% defibrinated sheep blood (Hema Resources) and 20ug/ml streptomycin (Sigma-Aldrich) for two days at 37ºC and stored at 4ºC. *K. aerogenes* was grown on plates of Luria-Bertani agar (Difco) for one day at 37ºC and stored at 4ºC.

Mouse experimentation

HeJ (TLR4 mutant C3) and HeN (wild-type C3) mice aged four to six weeks were obtained from Jackson Laboratory (Bar Harbor, ME) and kept in *Bordetella*-free rooms at Pennsylvania State University in cages of 3 or 4. C57BL6J (wild-type B6), µMt (B cell deficient B6), TCRβδ-/- (T cell deficient B6), and Rag-/- (T and B cell deficient B6) mice from Jackson Laboratories were also bred at the Pennsylvania State University facility.

Infections were performed with 100cfu of bacteria in 5ul of phosphate-buffered saline (PBS) applied to the nares after sedation with 5% isoflurane (Abbot Laboratories).

Shedding was monitored by a ten-second rubbing of the nares with Dacron-polyester tipped swabs. The tip of each swab was then vortexed for thirty seconds in 1ml PBS and dilution-plated onto BG+strep. Colonization of nasal cavities and lungs was measured by sacrificing mice, homogenizing in 1ml PBS, and dilution-plating onto BG+strep.

Neutrophil depletion

0.5mg of RB6-8C5 monoclonal antibody was delivered by intraperitoneal injection one day before infection, and every two days afterward during the experiment time course (Czuprynski, Brown, Wagner, & Steinberg, 1994). The depletion was confirmed by flow cytometry as discussed below.

Nasal neutrophil recruitment

After being sacrificed, 10-20ml of PBS was injected into the left ventricle of each mouse's heart, and drained from the eye socket. The nasal cavity was then dissected and the bones placed in 1ml Dulbecco's Modified Eagle Medium (Cellgro) with 5% fetal bovine serum (FBS) and 1mg/ml collagenase D (Roche). After incubating at 37°C for 45 minutes, a suspension of single cells was made by using mechanical disruption with a 70um mesh screen. Anti-CD16/32 (BD Biosciences) in PBS with 2% FBS Fc blocking buffer was used to suspend $2x10^6$ cells in each well of a 96-well plate, which was then put on ice for 20 minutes. After washing the blocking buffer, cells were labeled with APC anti-Ly6G (eBioscience) and anti-CD11b BD Horizon V450 (BD Biosciences) in PBS with 2% FBS and flow cytometry (Fortessa) was performed.

Blood neutrophil levels

After swabbing to record shedding, the facial vein of each mouse was pierced with a 21 gauge needle, and 25ul of blood was collected in a capillary tube and place in 10ul of 6% EDTA at pH 8. The blood/EDTA was then placed in .5ml of red blood cell lysis (.15M NH_4Cl, 10mM HCO_3^-) solution for 5 minutes at room temperature, then .5ml of cold PBS was added. The rest of the procedure leading to flow cytometry was performed as described above.

Amoeba cultures

After plating 200ul of mid-log phase food-source *K. aerogenes* on plates of SM-5 media and allowing to dry, freezer stock of *Dictyostelium discoideum* was swabbed and streaked over the entire plate. After approximately one week, fruiting bodies were removed with a sterile pipette tip and placed into 500ul of HL5 media. This was vortexed vigorously and added to 10ml of HL5 media with 500ug/ml streptomycin on a tissue culture plate. After two days, the media was replaced with HL5 (no streptomycin). Every three to four days afterward, the amoeba were passaged by removing media, resuspending in 1ml fresh HL5, and adding to a new plate containing 10ml HL5 (adapted from Fey, Kowal, Gaudet, Pilcher, & Chisholm, 2007).

Amoeba plate clearance assay

Bacteria were grown to mid-log density, adjusted to $1x10^9$cfu/ml, and then 200ul was plated onto SM-5. Freezer stock of amoeba was swapped, the tip of the swab was cut off and vortexed in 1ml of PBS. 10ul of this mix was dropped onto each SM-5 plate, and the location of the spot recorded by marker on the bottom of the plate.

Over the time course, clearance of the bacterial lawn was observed as the amoeba spread outward from the marked spot. The distance was measured to the point of clearance furthest from the spot to gauge how far the amoeba had spread.

Intracellular survival assay

When the amoeba appear relatively confluent on the plate, the media was removed and the amoeba resuspended in 1ml HL5 media. This was then added to additional HL5 up to a total volume of 10ml and then distributed 100ul to each well on a 96-well plate (except for the outermost wells, which should receive HL5 without amoeba due to evaporation, and wells for the gentamicin controls).

After overnight incubation at room temperature, one of the wells was counted using a hemocytometer (however, it was no longer be usable for the experiment). For a multiplicity of infection of 50, bacteria were brought to a concentration such that in the 10ul added to each well, there were 50 times as many bacteria as amoeba already in the well. The 96 well plate was centrifuged at 1500 rpm for 5 minutes at room temperature, then incubated for one hour at room temperature to allow the amoeba to phagocytize the bacteria. At this point (t=0), media was removed from all wells (with the exception of the triton controls) and replaced with 100ul of 100ug/ml gentamicin in HL5 media.

At t=0, after one hour of incubation of bacteria and amoeba, the triton control wells had 100ul of .1% Triton X in HL5 added, and after five minutes incubation at room temperature, were spot plated (8 drops of 10ul, each diluted 10 times from the previous) onto the appropriate media (BG+strep for *B. bronchiseptica* and LB for *K. aerogenes*). This number was used as 100% bacterial survival. To ensure total killing by the gentamicin, the appropriate number of

bacteria was added to 100ul of HL5+gentamicin and spot plated after one hour incubation at room temperature. At t=0 and t=24, HL5+gentamicin was removed from wells. Wells were washed three times with HL5 (no gentamicin), incubated with 100ul Triton X for five minutes, and then spot plated.

Results

Bordetella transmits efficiently between TLR4 deficient mice

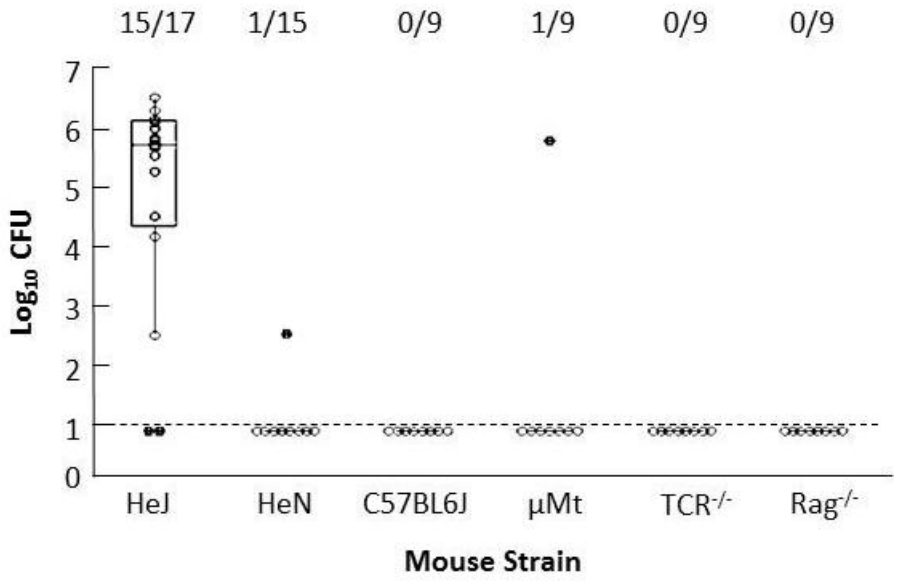

Figure 1: Nasal colonization of secondary case mice exposed to index case after 4 weeks

In order to ascertain how different immune components are necessary to limit transmission of *Bordetella bronchiseptica*, six different types of mice were infected with 100cfu each of *B. bronchiseptica* to act as index cases in a transmission study. Each index case was placed with either two or three uninfected cagemates of identical genotype. These different genotypes were HeJ (TLR4 mutant C3), HeN (wild-type C3), C57BL6J (wild-type B6), µMt (B cell deficient B6), TCRβδ-/- (T cell deficient B6), and Rag-/- (T and B cell deficient B6). After four weeks, the secondary cases were sacrificed and their nasal cavities cultured for the presence of *B. bronchiseptica*.

Results showed that out of 17 potential secondary cases of HeJ mice, 15 had been infected, while in HeN mice, only 1 out of 15 potential secondary cases had been infected. In all

of the B6 mice (which were either wild-type, B cell knockout, T cell knockout, or Rag-/-) there was only a single transmission event, in the B cell knockout mice.

The results show that TLR4 is necessary to prevent transmission. TLR4 is important in recognizing LPS of gram-negative bacteria and initiating the innate response, so the suggestion is that the innate immune system is more important in controlling transmission than the adaptive immune system, since B and T cell knockouts (with compromised adaptive immunity) did not have significant transmission.

TLR4 is required to limit bacterial shedding

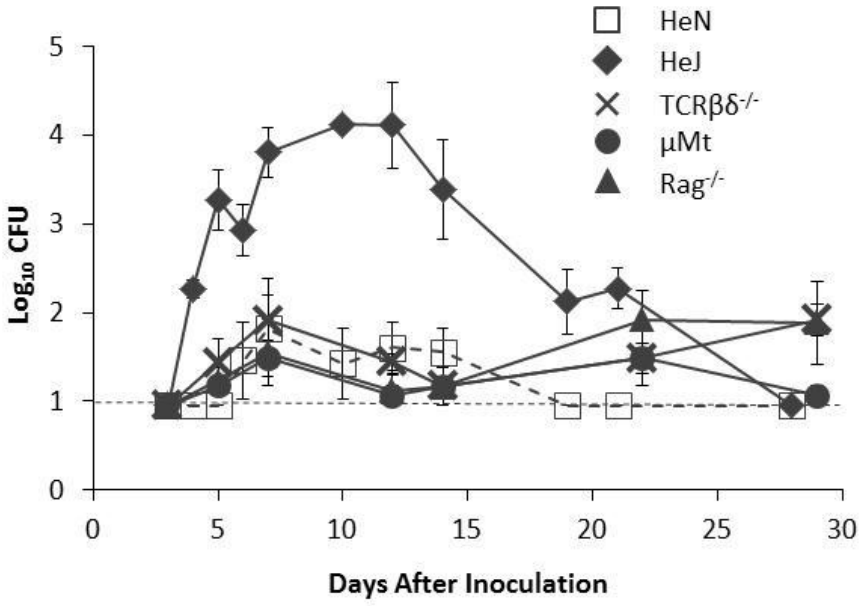

Figure 2: Bacterial shedding from nares of infected mice

Since increased transmission can be either due to increased shedding, increased susceptibility, or both, we measured the bacterial shedding from HeJ (TLR4 mutant C3H), HeN (wild-type C3H), µMt (B cell deficient B6), TCRβδ-/- (T cell deficient B6), and Rag-/- (T and B

cell deficient B6) mice. In groups of three or four, each mouse was infected with 100cfu of *B. bronchiseptica* and a ten second nasal swab was done on each mouse at several timepoints over a period of 28 days.

We found that shedding from HeJ mice was significantly higher than shedding from wild type HeN mice. At the peak of shedding for HeJ, levels are over 100 times higher than in the HeN mice. While the HeN mice did not shed at detectable levels after day 18, shedding in HeJ mice was detectable until day 28. This suggests that the TLR4 receptor and its ensuing innate immune response is important in controlling shedding during the early stages of infection and limiting the duration of the infection.

Shedding in, μMt, TCRβδ-/-, and Rag-/- mice peaked at day 7 before decreasing, the same trend as with the wild type HeN mice. The difference in shedding between these is not significant up to day 14. After this, HeN shedding sinks below the limit of detection, most likely because of adaptive immunity taking control of the infection. However, the mice with compromised adaptive immunity shed at increasing levels until day 22, and then at detectable levels until the end of the experiment at day 28 post-infection. This seems to confirm that adaptive immunity is necessary for longer-term control of shedding, while innate immunity is more important for early control.

TLR-4 is important in controlling lung and nasal colonization

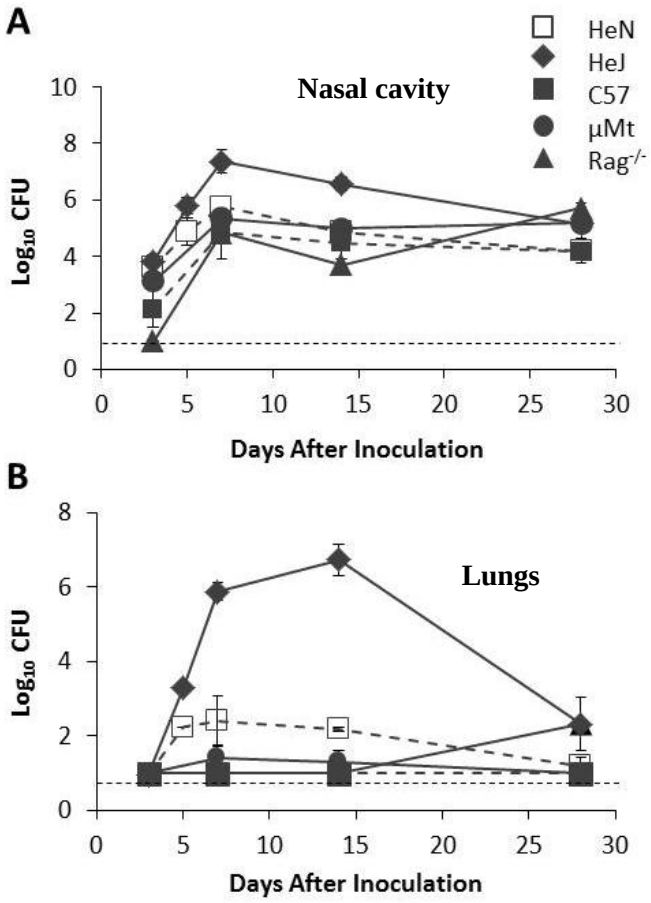

Figure 3: Colonization of nasal cavity (A) and lungs (B) of infected mice

The second piece to understand the transmission puzzle in addition to shedding is host susceptibility. In order to assess this, we infected HeJ, HeN, C57BL6J, µMt, and Rag-/- mice with 100cfu of *B. bronchiseptica*. In groups of 4, they were sacrificed at days 3, 5, 7, 14, and 28 to culture the lungs and nasal cavities for *B. bronchiseptica* colonization levels.

At day 7, the peak colonization in the lungs, there were nearly 1000 times more bacteria in the lungs of the HeJ as compared to the wild-type HeN. At day 14, the peak lung colonization of HeJ mice, there were nearly 10,000 times more bacteria in the lungs of the HeJ mice than in the HeN mice. In contrast, there was no significant difference between the wild-type HeN and

B6 mice and the adaptive-compromised μMt and Rag-/- mice in lung colonization. This suggests that TLR4 and the innate response is important in limiting the bacterial burden in the lungs more so than the adaptive immune response. Towards the end of the time frame, the numbers of bacteria in the lungs of Rag-/- mice appears to be rising, which may indicate a necessity for B and T cells in limiting the infection long-term in the lungs.

In the nasal cavity, the same trend was shown. At the day 7 peak of infection, colonization is about 20 times higher in HeJ compared to the wild-type HeN, showing that nasal colonization levels are also controlled by the presence of TLR4, although not as dramatically as in the lungs. At the end of the time frame, all of the colonization numbers are sinking except for the Rag-/- mice, once again showing the necessity of adaptive immunity for long-term control. TLR4 deficient mice shed at higher levels (Figure 2), and they also appear to be more susceptible to colonization, as indicated by the higher levels of bacterial burden post-infection.

TLR4 reduces susceptibility to infection from infectious hosts

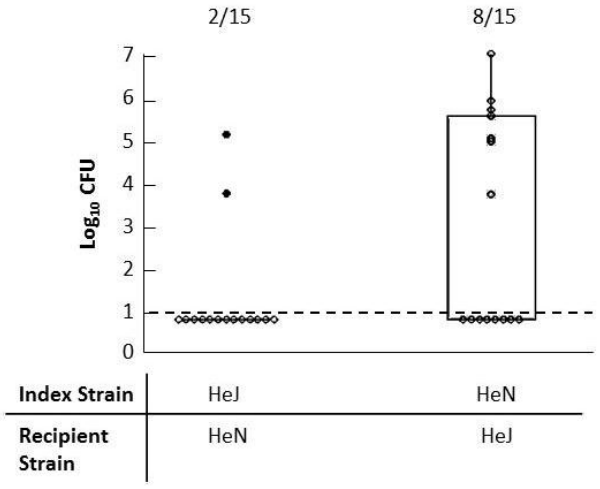

Figure 4: Nasal colonization of secondary case mice exposed to index case after 3 weeks

In order to confirm the relative susceptibilities of mice with and without the TLR4 receptor, uninfected HeJ mice were caged with HeN index cases and vice versa. 8 out of 15 potential secondary HeJ mice were infected by the HeN index cases, while only 2 out of 15 HeN were infected by the HeJ index cases.

Figure 2 shows that HeJ mice shed at higher levels than HeN mice, so the shedding of the HeN index case was not the cause of the higher transmission to HeJ mice. Therefore, in addition to Figure 3 showing that HeJ mice are more susceptible to higher bacterial growth within the host, Figure 4 shows that HeJ mice are also far more susceptible to becoming recipients of a transmission event, even from a relatively low-shedding index case.

Higher levels of shedding correspond to higher levels of neutrophils

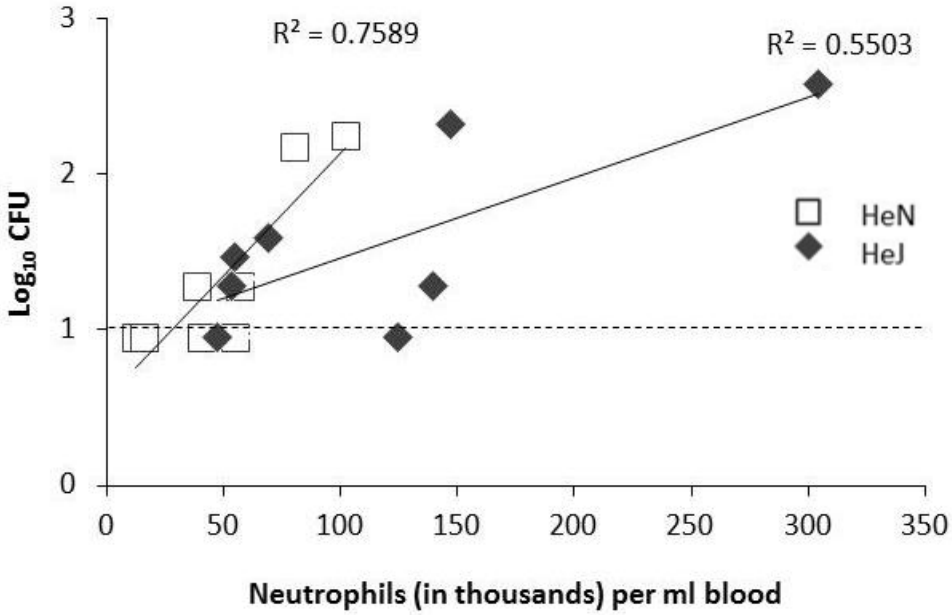

Figure 5: Neutrophil and shedding levels at day 7 after infection

To understand which immune cells were involved in controlling shedding, a blood panel was done that revealed a high correlation between shedding levels and levels of neutrophils in the blood of the mice. To conduct this analysis, at day 7 after infection, murine nares were swabbed for ten seconds to measure shedding, and this was immediately followed by blood collection from a facial vein.

The results showed a high level of correlation between shedding and neutrophil levels in the blood both in HeN and HeJ mice. The correlation is stronger in the wild-type HeN mice, suggesting that the correlation of shedding to neutrophil levels may for some reason be altered in the absence of TLR4.

Shedding increases significantly one week after neutrophil depletion

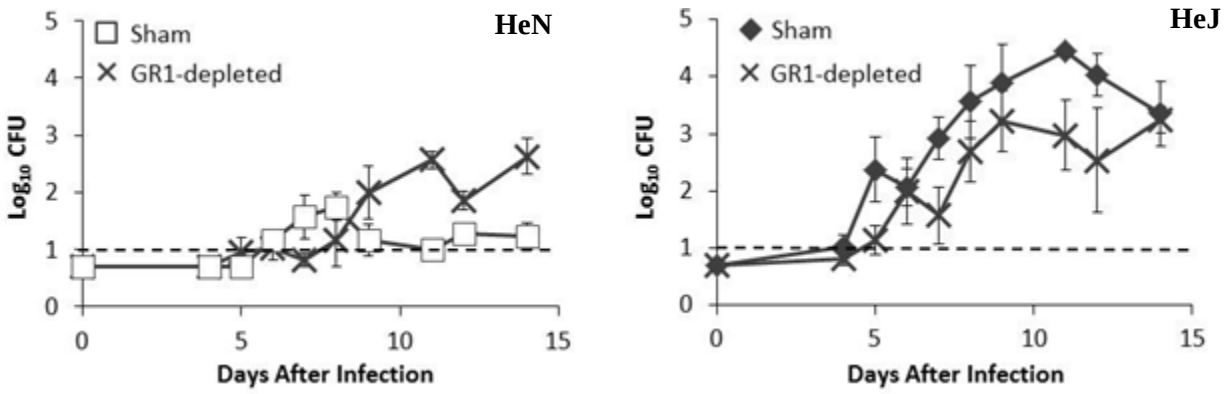

Figure 6: Levels of shedding in HeN (A) and HeJ (B) mice during neutrophil depletion

In order to see the effects of neutrophil depletion on shedding, HeN and HeJ mice were given an intraperitoneal injection of either control ("sham" on Figure 6) IgG2b antibody or anti-Gr-1 antibody for depletion of neutrophils every other day for 14 days. After the first day of

depletion, the mice were infected, and then were monitored for shedding throughout the time course by a ten second swab of the nares.

The results show that, at the beginning of the time course in HeN mice, shedding is below the limit of detection for neutrophil-depleted mice as shedding in the mice without depletion climbs. However, at day 7, shedding in the neutrophil-depleted mice suddenly spikes and overtakes the shedding numbers for the non-depleted mice, remaining at higher levels for the rest of the time course. A similar delayed spike is observed in the HeJ mice, without such a dramatic overtake of the shedding numbers of non-depleted mice by the depleted mice (however, the non-depleted HeJ mice already had much higher shedding levels in comparison to the non-depleted HeN mice. See figure 2).

There are two potential explanations for the change in shedding. First, by some effect of neutrophil depletion, nasal colonization could climb quickly in the neutrophil-depleted mice around day 7, resulting in higher shedding. Second, and our hypothesis, was that some change in neutrophil numbers around day 7 causes the higher shedding, in an extension of the trend seen in Figure 5.

Neutrophil rebound after depletion results in higher shedding

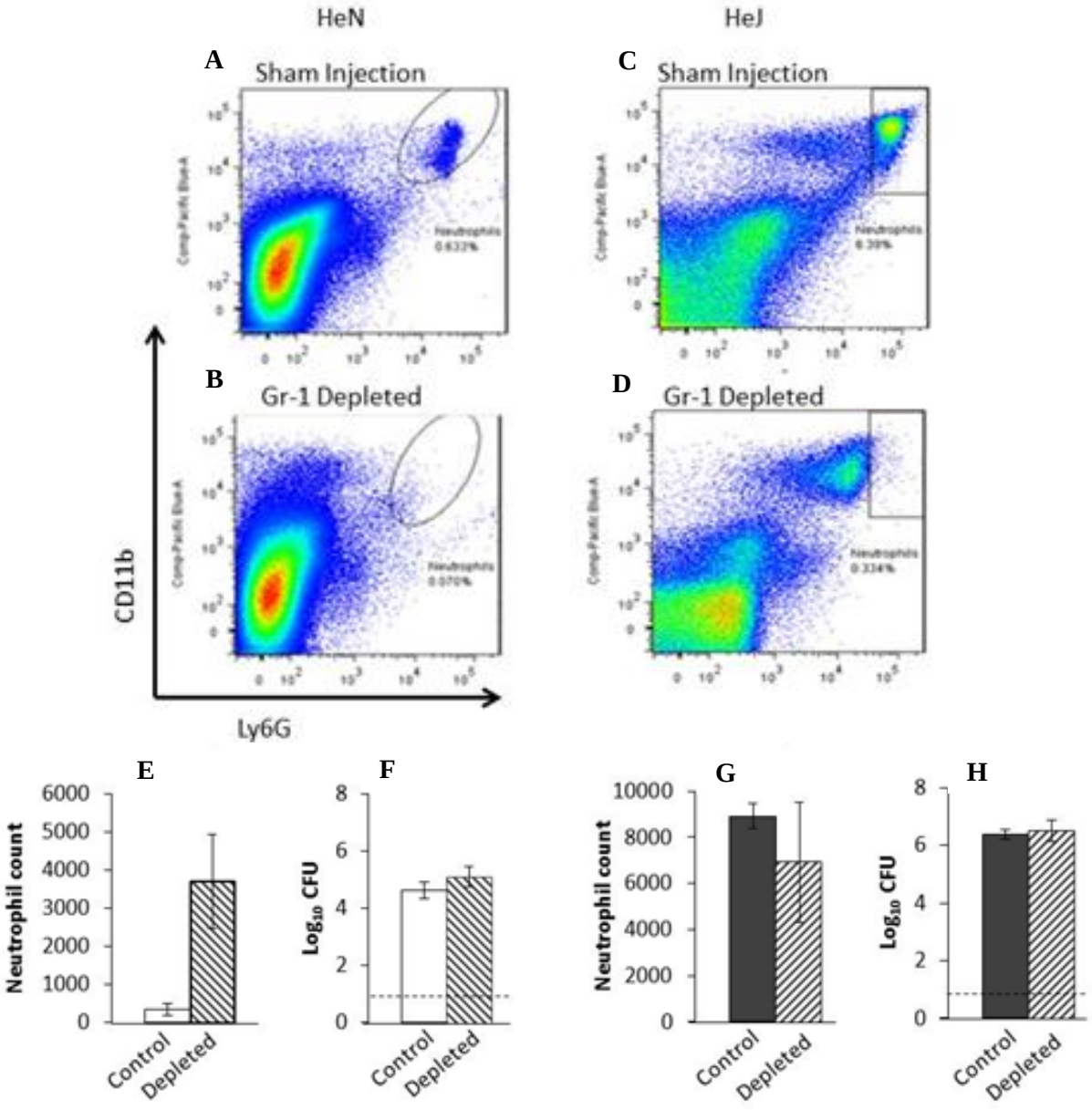

Figure 7: Flow cytometry for neutrophils in nasal cavities of control HeN (A), neutrophil depleted HeN (B), control HeJ (C), and neutrophil depleted HeJ (D) at 7 days. Neutrophil counts in nasal cavities of HeN mice (E) and HeJ mice (G) at 14 days. Nasal colonization for HeN mice (F) and HeJ mice (H) at 14 days.

To further elucidate the reason for the change in shedding in the neutrophil depleted mice, we performed flow cytometry for neutrophils day 7 after start of depletion (A-D above). The relative presence of neutrophils in the control populations (depicted by the oval in A for HeN and box in C for HeJ) and the relative absence of these neutrophils in the depleted populations (depicted by the oval in B for HeN and the box in D for HeJ) was observed as expected. This shows that at day 7, the neutrophil depletion is successful. Correspondingly, at this point in Figure 6, shedding has not yet appreciably begun in the HeN mice. However, in the HeJ mice, shedding is just starting to increase, but this could be attributed in some way to the population of cells just left of the box in D, which we suspect are monocytes recruited as part of a mechanism unique to HeJ mice.

At day 14, when the shedding data in Figure 6 has shown the significant increases in both the HeN and HeJ mice, we quantified the number of neutrophils to compare levels between the control and depleted mice. In the HeN mice (E), the numbers of neutrophils are far higher in the originally successfully neutrophil depleted population. In the HeJ mice (G), there is no significant difference between neutrophils in the depleted and control population. However, in comparison to the HeJ day 7 flow cytometry data showing an absence of neutrophils, there clearly has been a spike in the depleted population. As previously stated, the similarity of the neutrophil levels in the depleted and control at this point could be explained by the abnormally high numbers of neutrophils in the control population due to the high numbers of bacteria. If the levels in the control were not so high to begin with, it might be expected that the neutrophil numbers in the depleted population would have surpassed that of the control population. This all suggests that higher neutrophil numbers, as indicated by the change from being absent in day 7

to abundant at day 14 compared to the controls, explains the intense increase in shedding in the neutrophil depleted mice.

The alternative explanation is that the nasal colonization is higher in the neutrophil depleted mice, which would contribute to higher shedding towards the end of the time frame. However, this is clearly not the case. Figure 8F shows there is no significant difference in colonization in HeN at day 14, while neutrophils are far higher in the depleted population. Figure 8H shows no significant different in colonization in the HeJ mice, so again, the rapid rise in shedding is attributable to the rise in neutrophil numbers.

D. discoideum growth is slow in presence of *B. bronchiseptica*

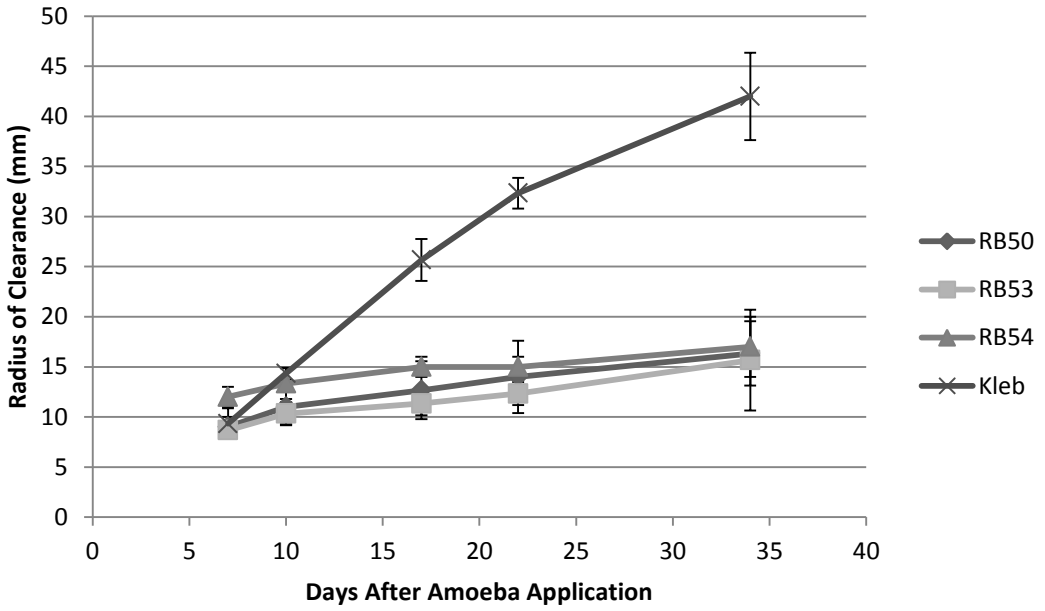

Figure 9: Largest radius of clearance of RB50, RB53, RB54, and *K. aerogenes* by *D. discoideum*

To investigate the possibility of *D. discoideum* functioning as a potential environmental reservoir for transmission of *Bordetella*, we looked at the ability of the amoeba to survive in the presence of *B. bronchiseptica*. After plating 200ul of mid-log phase RB50 (wild-type *B.*

bronchiseptica), RB53 (Bvg+ phase-lock mutant RB50), RB54 (Bvg- phase-lock mutant RB54), and *K. aerogenes*, we added 10ul of a suspension of amoeba spores and marked the location of this drop. For the next 34 days, the growth of the amoeba was recorded by measuring the largest radius of the zone of bacterial clearance around the spot of amoeba application.

There were no significant growth differences of *D. discoideum* on the plates containing different strains of *B. bronchiseptica*; however, the amoeba spread significantly faster on the *K. aerogenes* (its typical food-source) plate compared to any of the plates containing *B. bronchiseptica*. Also, while spread of the amoeba was slowed on each of the *B. bronchiseptica* species compared to *K. aerogenes*, it was not totally inhibited. This shows that *Bordetella* is most likely not as efficient a food source for the amoeba as *K. aerogenes* is, but also appears not to be completely pathogenic to the amoeba. Since the different *B. bronchiseptica* phases result in similar growth of *D. discoideum*, it appears that the expression of virulence factors in the Bvg+ phase does not significantly affect the spread and growth of the amoeba.

Bvg phased-locked and wild-type *B. bronchiseptica* are phagocytized similarly by *D. discoideum*

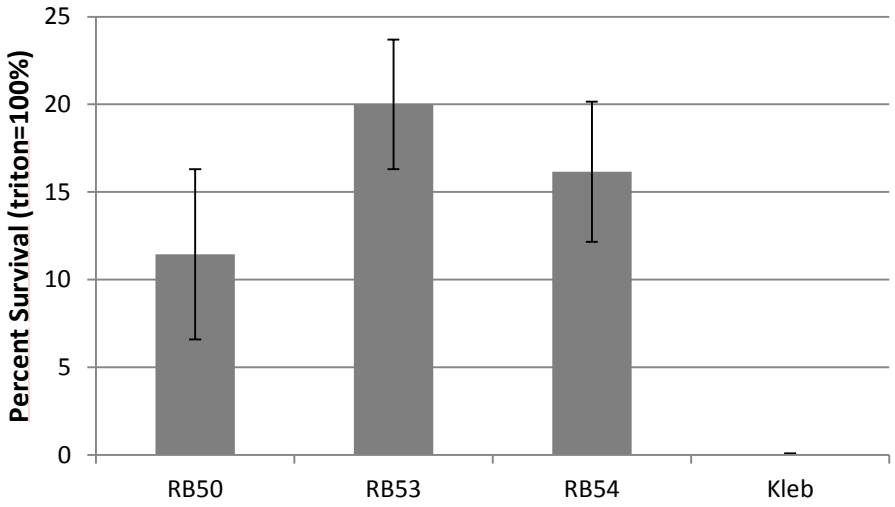

Figure 8: Recovery of RB50, RB53, RB54, and *K. aerogenes* after one hour in *D. discoideum*

To see if *B. bronchiseptica* can survive intracellularly in *D. discoideum* as it does in macrophages, we infected the amoeba with RB50, RB53, RB54, and *K. aerogenes* as a negative control, since *Dictyostelium* digests it so efficiently that it does not survive intracellularly to an appreciable extent (Froquet, Lelong, Marchetti, & Cosson, 2008). After allowing the amoebae and bacterial cells to combine for one hour, they were gentamicin treated so that the only bacterial cells left were ones surviving intracellularly within the amoebae. Then, after another hour, triton was used to lyse the amoeba cells via vigorous pipetting, which caused the release of bacteria for counting. Bacteria released from amoeba lysed with triton without gentamicin treatment represents 100% survival.

Since only one hour had passed between combining the bacteria and amoeba and adding gentamicin, the bacteria recovered from the amoeba are more of a measure of the extent to which they are phagocytized than a measure of extended intracellular survival. After one hour in the amoeba cells, almost no *K. aerogenes* was recovered, as expected. Among the *B. bronchiseptica*

bacteria, there was no significant difference in bacterial recovery at this time point. The difference between RB50 and RB53 was insignificant (p=.072), and the difference between RB50/RB54 and RB53/RB54 was even smaller, showing that all Bvg phases of *B. bronchiseptica* are phagocytized by the amoeba to a similar extent.

The Bvg- phase is necessary for intracellular survival for 24 hours in *D. discoideum*

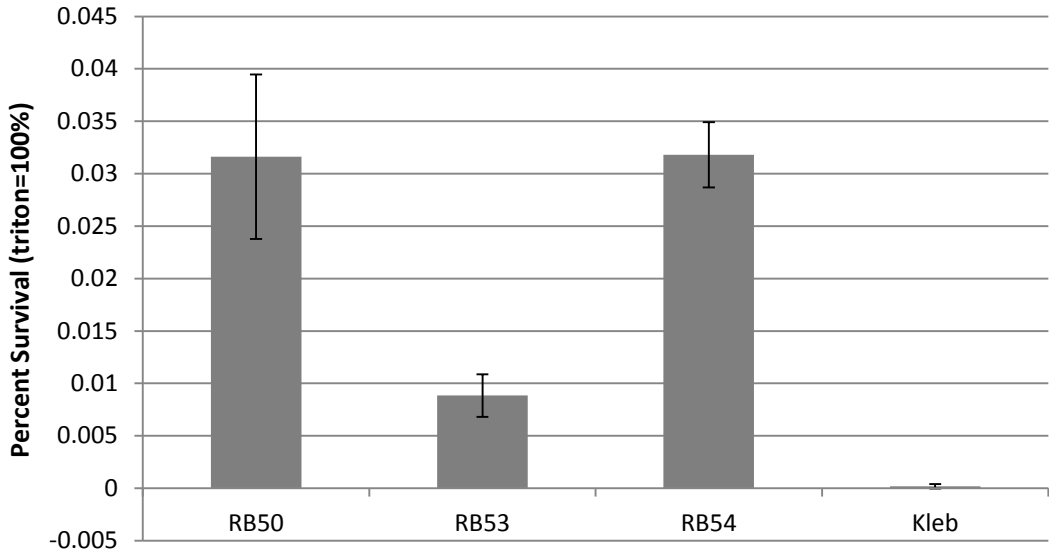

Figure 9: Intracellular survival of RB50, RB53, RB54, and *K. aerogenes* for 24 hours in *D. discoideum*

Following the same protocol as before, we also lysed the amoeba cells 24 hours after gentamicin treatment. This time, while there was no difference between RB50 and RB54, they both are significantly higher than RB53, the Bvg+ phase-locked mutant.

Discussion

Understanding host immune responses is important in understand how to control transmission of infectious diseases because these responses determine both the susceptibility of the individual to catching diseases and its tendency to shed infectious microbes to perpetuate disease in others (Pathak, Creppage, Werner, & Cattadori, 2010). In examining the roles of innate and adaptive immunity in controlling transmission of *B. bronchiseptica* in mice, we found that innate immunity, triggered by TLR4 was important in controlling susceptibility, shedding, and transmission.

The finding that *B. bronchiseptica* only readily transmitted through a TLR4 deficient population, as opposed to wild-type and B or T cell knockouts, showed that innate immunity is important in controlling transmission. Delving deeper to discover whether this is due to increased shedding among TLR4 deficient mice or because of increased susceptibility, we found that both were in fact true. Shedding was much higher in the TLR4 deficient mice and took 28 days to bring below detectable levels, compared to 18 days in the wild type mice. Colonization of the nasal cavity and especially in the lungs of TLR4 deficient mice was far higher, indicating a great susceptibility. Also, susceptibility was proven to be much higher in the TLR4 deficient mice because many more TLR4 deficient mice caught *B. bronchiseptica* from the low-shedding wild type mice than vice versa.

Towards the end of the time course in the lung and nasal colonization experiment, bacterial numbers in the Rag-/- mice seems to indicate that the combination of T and B cells is required for long term control of bacterial numbers. This is also suggested by the shedding data, where wild-type and TLR4 deficient mice (who still have their adaptive immunity intact) have

the infection reduced below detection levels while T cell, B cell, and Rag deficient mice still have noticeable levels. Further experimentation would need to be done on a longer time course to show the importance of adaptive immunity in controlling infection.

We found that an important part of the immune system which contributes to greater infectiousness is the presence of neutrophils. In a panel correlating levels of different immune cells in the blood to shedding levels, the only clear correlation was with neutrophils. Investigating further, we found that neutrophil depleting mice delayed the onset of bacterial shedding until the depletion failed, and the rebound in neutrophil levels happened at the same time as a sharp increase in bacterial shedding. This increase of shedding had nothing to do with an increase of nasal colonization, as the colonization was the same between the control and the neutrophil-depleted groups. What is unclear so far is the mechanism explaining this trend; we have hypothesized that the neutrophil response might increase the output of mucus containing microbes, increasing the likelihood of other individuals coming in contact with infectious droplets. This is based on the observation in a study on COPD patients which showed that the neutrophil responses cause goblet cells to degranulate and increase mucus production (Nadel, 2000). However, further research would need to be done to confirm that the same mechanism is acting during *B. bronchiseptica* infection in mice. Additionally, it would be important to explore the implications on the development of disease in an individual lacking neutrophils to see if neutrophil depletion is a reasonable step to take to decrease infectiousness of that individual.

Our studies of *D. discoideum* showed, at least preliminarily, that it has the potential to be an environmental reservoir of *B. bronchiseptica,* and therefore potentially also of the human pathogen *B. pertussis*. First, we observed the ability of the amoeba to grow in the presence of *B. bronchiseptica*. While *K. aerogenes* is readily consumed by amoeba as a food source and

correspondingly the zone of clearance grows quickly, the fact that the zone of clearance of all *B. bronchiseptica* types is considerably less suggests that they are not a food source to the extent that *K. aerogenes* is. Another study showed that amoeba which grow quickly on a lawn of bacteria are typically able to consume and digest those bacteria easily (Akya, Pointon, & Thomas, 2010), so this is not the relationship between *D. discoideum and B. bronchiseptica*. Since *D. discoideum* is extremely phagocytic (Cosson & Soldati, 2008), there appeared the possibility of *B. bronchiseptica* being consumed but able to persist intracellularly without being destroyed, especially in light of studies showing its ability to survive in human macrophage cells (Lamberti, Hayes, Perez Vidakovics, Harvill, & Rodriguez, 2010). Indeed, we showed that *B. bronchiseptica* was phagocytized by the amoeba (with no significant difference between different Bvg phases), and also that after 24 hours, the less-virulent Bvg- phase had significantly higher intracellular survival than the virulent Bvg+ phase, indicating that this phase would be more likely to encounter in the environment. This appears logical, since this phase is adapted for the purpose of survival by *Bordetella* when placed in a less-than-ideal environment (Cotter & Miller, 1994). This is contrasted to the conclusion of Caeiro et al, who predicted that more virulent strains might be more likely to survive, a scenario which could potentially be more dangerous (Caeiro, et al., 2012). In order to ascertain whether the survival within amoeba is truly a plausible method of transmission, a thorough *in vivo* study needs to be done to see if infected amoeba can transmit *B. bronchiseptica* to a murine host.

Our results in studying *B. bronchiseptica* have shown a number of different directions to be explored as potential ways to interrupt the chain of transmission of *B. pertussis* in humans. Since the innate immune system is the most important part involved in immediate control of the infection and its shedding, perhaps a therapy could be developed that would strengthen the innate

response, just as vaccines strengthen the adaptive response. In the case of someone who already has the illness, in order to protect their family and friends, a therapy to reduce their neutrophil response and therefore shedding until the infection has run its course would be valuable. Finally, in protecting others from the potential persistence of *B. pertussis* in an amoeba environmental reservoir as appear possible with *B. bronchiseptica*, simple disinfectants cannot be the only ones used in areas such as hospitals: steps also need to be taken to eliminate the amoeba protecting their intracellular bacteria. Until an effective vaccine is developed and becomes widespread, further research into transmission of *B. bronchiseptica* may be the best chance we have at controlling the spread of *B. pertussis*.

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EDUCATION

The Pennsylvania University, Schreyer Honors College — Expected May 2013
Bachelor of Science in Immunology and Infectious Disease — MCAT: 36R
Minor in Global Health, Certificate in Humanitarian Engineering and Social Entrepreneurship

RESEARCH

Microbiology Lab of Dr. Eric Harvill, Penn State University — 10/2010 - present
Research Assistant

- Paper submitted to Infection and Immunity: "Toll Like Receptor 4 Limits Transmission of *Bordetella bronchiseptica*" (third author)
- Perform experiments on transmission and intracellular survival of respiratory pathogens

Humanitarian Engineering and Social Entrepreneurship program, Penn State University

- Redesigned press to construct affordable, sustainable ceramic water filters
- Henry, M., S. Maley, and K. Mehta. 2013. Designing a Low-cost Ceramic Water Filter Press. *International Journal for Service Learning in Engineering* (accepted with revisions 3/22/13)

Immunology Lab of Dr. Christoph Wuelfing, University of Texas Southwestern Medical Center — Summer 2011
Summer Undergraduate Research Fellow

- Poster presentation: "Nck Adapter Protein Localization and T Cell Activation"
- Used molecular techniques to clone modified DNA and 3D microscopy to study T cells

ACTIVITIES

Founder and President, Global Brigades Campus Council at Penn State — 8/2012 - Present

- Coordinate activities of several hundred members between seven different Brigades clubs on campus
- Act as liaison to national Global Brigades USA nonprofit organization

Co-Founder and Co-President, Global Water Brigades, Penn State Chapter — 8/2010 - 5/2012

- Started club in August 2010
- Led total of 64 students to Honduras and Ghana to work on clean water systems
- Worked on campus to fundraise, recruit, and raise awareness of water crisis
- As member of National Water Brigades Advisory Council, worked on program planning for all Water Brigades groups nationally

Penn State Cycling Club — Fall 2011 - Spring 2013

- D classification road racer
- C classification mountain racer

Saxophonist in Penn State Concert and Jazz Bands — 1/2010 – 5/2011
Penn State Dance Marathon Rules and Regulations Committee — 9/2009 -5/2010

RELATED EXPERIENCE

Mount Nittany Medical Center Volunteer — 6/2010 – 12/2011
Global Health Minor Fieldwork in Tanzania — Summer 2012
Centre Volunteers in Medicine Volunteer — 9/2009 - 4/2010

WORK EXPERIENCE

Food Service, Warnock Commons at North Halls, University Park, PA — 1/2013-present
Lifeguard, Indian Creek Recreational Club, Mechanicsburg, PA — 6/2006 – 8/2008

HONORS AND AWARDS

Parmi Nous Honor and Tradition Society — Spring 2012
Ralph Dorn Hetzel Memorial Award — Spring 2013
Penn State Rhodes and Marshall Scholarships Nominee — Fall 2012
Horace T. Woodward Scholarship from College of Agricultural Sciences — Fall 2012
Milking the Rhino Innovative Solutions Showcase: Best Video Pitch Award — Fall 2012
Steele Fund travel grant for Tanzania Fieldwork — Summer 2012
Schreyer Honors College Research Grant — Summer 2012
USA Today Leadership Award — Spring 2012
Service Learning Award — Spring 2012
College of Agriculture Research Grant — Spring 2012
Schreyer Honors College travel grants (5 total)

- 1 Medical and 2 Water Brigades to Honduras, Water Brigade to Ghana, Fieldwork in Tanzania

North Mechanicsburg Rotary Club Student of the Year — Spring 2009
North Mechanicsburg Rotary Club leadership conference full scholarship recipient — Summer 2008