The Pennsylvania State University

The Graduate School

Department of Biochemistry, Microbiology, and Molecular Biology

INTERLEUKIN-6 CONTRIBUTES TO IMMUNITY AGAINST *BORDETELLA PERTUSSIS*

A Thesis in

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by

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ABSTRACT

Bordetella pertussis, an endemic human respiratory pathogen, causes whooping cough, a severe coughing illness. The number of cases of whooping cough is increasing despite high vaccination coverage. In the last decade, the function of leukocytes and cytokines during *B. pertussis* infection has been characterized. Here, we investigate the effect of a pro-inflammatory cytokine, interleukin (IL)-6, on clearance of this respiratory pathogen using a murine model of infection. *B. pertussis* cleared from the lungs of wild-type mice approximately 40 days postinoculation. However, in the absence of IL-6, *B. pertussis* grew to higher numbers and persisted more than 77 days. Also, IL-6 contributed to infection-induced immunity. *B. pertussis* specific serum antibody titers were decreased in the absence of IL-6, most of which were T cell dependent antibody subtype, IgG2b. Splenic T cell cytokine responses were dampened in helper T cell (T_H) 1 and T_H2 cytokine production in response to *B. pertussis*. $CD4^+$ T cells and neutrophils, both required to clear *B. pertussis* infection, were found to be IL-6 dependent for recruitment to the lungs. Together, these results indicate that IL-6 contributes to clearance of *B. pertussis* in both naïve and previously infected hosts. Delay in clearance of this bacterium is likely caused by a defective T-cell response in the absence of IL-6.

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LIST OF ABBREVIATIONS

CFU: colony forming unit

DMEM: Dulbecco's modified Eagle cell culture medium

ELISA: enzyme linked immunosorbent assay

FHA: filamentous hemagglutinin

GM-CSF: granulocyte-macrophage colony stimulating factor

IACUC: institutional animal care and use committee

IFN: interferon

Ig: immunoglobulin

IL: interleukin

i.p.: intraperitoneal

MOI: multiplicity of infection

PBS: phosphate buffered saline

Ptx: pertussis toxin

T_H: T helper

TNF: tumor necrosis factor

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CHAPTER 1: INTRODUCTION

Bordetella pertussis is a gram negative bacterial pathogen which is one of the etiologic agents of an acute and severe disease, whooping cough. It colonizes the human respiratory tract causing the severe coughing illness which can progress into paroxysmal coughing [1]. There were ~50 million cases and > 300,000 deaths due to whooping cough worldwide, in 2002 [2]. Despite the widespread use of vaccines, there is a rise in the number of individuals transiently infected with *B. pertussis* in immunized populations [3-6]. Current pertussis vaccines induce a strong serum antibody response which has been shown to be critical for protection against *B. pertussis* [7, 8]. The T cell response varies from a balanced T_H1/T_H2 response to a T_H2 skewed response with the type of vaccine in use [9-11].

B. pertussis synthesizes and secretes many adhesins and toxins which are involved in its pathogenicity. Adhesins such as filamentous hemagglutinin (FHA), fimbriae, and pertactin, an autotransporter, are used by the bacterium for attachment to the eukaryotic respiratory epithelium [12-14]. *B. pertussis* produces many toxins upon infection such as pertussis toxin (Ptx), adenylate cyclase-hemolysin, and tracheal cytotoxin [15-17]. Studies in our lab along with others have established the role of various host immune factors such as B cells, antibodies, Fcγ receptors, neutrophils, $CD4^+$ T cells, TNF-α and IFN-γ in immunity against *B. pertussis*. B-cell deficient mice are unable to clear *B. pertussis* from the respiratory tract establishing the requirement of antibodies in bacterial clearance [18-20]. Passive transfer of *B. pertussis* serum specific antibodies is able to clear the bacteria from the murine respiratory tract between 7 and 14 days postinoculation via Fcγ receptors and neutrophils [21]. The adoptively transferred serum antibodies begin to affect bacterial numbers around the time when T cell responses to *B. pertussis* become detectable [22]. Initial studies have established $CD4^+$ T cells and T_H1 type responses to be protective against *B. pertussis* [10, 19]. Recently, the function of cytokines such

as TNF-α [23] and IFN-γ [unpublished data, Wolfe D.N.] has been characterized during *B. pertussis* infection.

Interleukin (IL)-6 is a classic multifunctional cytokine that was first cloned in 1986 [24]. It has various biological functions within the host and stimulates a variety of cell types. IL-6 is readily detected in the serum during stress conditions and inflammatory response [25]. A variety of cell types like monocytes, macrophages, endothelial cells, and T cells produce it. During acute inflammatory response, monocytes and macrophages are a major source of this cytokine whereas T cells are a primary source during chronic inflammation [26-30]. This cytokine signals in two ways, one by interacting with its receptor on the cell surface of neutrophils, monocytes, T cells and B cells [31], and the second by binding to its receptor in the soluble form and transducing signals on any cell with the signal transducing receptor, gp130, on its surface [32].

IL-6, though first produced as a part of innate immune response, plays a crucial role in the host's adaptive immune responses. Along with its soluble receptor (sIL-6Ra), IL-6 has been shown to activate production of some chemokines such as MCP-1 and IL-8 by endothelial cells which upregulates expression of adhesion molecules and thus contributes to recruitment of leukocytes at the site of inflammation [33]. It plays a critical role in differentiation of B cells and promotes proliferation of plasmablasts during their final stages of maturation into immunoglobulin producing plasma cells [34-37]. IL-6 also stimulates T cells [38] and recent reports have established a role of this cytokine in guiding naïve T cells to T_H17 cells, a lineage shown to be involved in development of autoimmune diseases [39-41]. The extensive research is helping to establish IL-6 as an interface cytokine between innate and adaptive immune response.

IL-6 deficient mice (IL-$6^{-/-}$) were first reported in 1994 [42]. Deficiency of IL-6 has been shown to cause defect in T_H1 regulated antibody and T_H1 cytokine production. Defects in

cytotoxic lymphocyte function and neutrophil production have also been reported in the absence of IL-6 [42-44]. IL-$6^{-/-}$ mice have been shown to be inefficient in controlling vaccinia virus as well as have impaired immune response against vesicular stomatitis virus [42]. Deficiency of IL-6 has been shown to impair host defense to many other pathogens such as *Listeria monocytogenes* by inefficient neutrophilia [45], *Borrelia burgdorferi* by decreased T_H2 responses [46], and *Chlamydia trachomatis* by decreased T_H1-like response [47]. IL-6 is also produced upon interaction between host respiratory epithelial cells and virulence factors of respiratory pathogens such as *Pseudomonas aeruginosa,* and *Haemophilus influenzae* [48-51]. Additionally, impaired defense against pneumococcal pneumonia has been reported in the absence of IL-6 upon infection with *Streptococcus pneumoniae* [52].

Since IL-6 is known to be produced in response to various respiratory pathogens [49-52], and is crucial for host immune response, we sought to determine its role in protection against *B. pertussis*. In this study we show that IL-6 contributes to clearance of *B. pertussis* from murine lower respiratory tract and there was a delay in clearance of *B. pertussis* in its absence. A defect in infection-induced immunity to *B. pertussis* in IL-$6^{-/-}$ mice led us to investigate the role of IL-6 in aiding the antibody response against *B. pertussis*. The absence of IL-6 appears to modulate the immune response by decreasing the number of *B. pertussis* specific, T cell dependent antibodies (IgG2b). A dampening of splenic T_H1 and T_H2 cytokine production was observed in IL-$6^{-/-}$ mice. Further, we investigated effect of IL-6 on leukocyte recruitment to the lungs and found $CD4^+$ T cell and neutrophil recruitment in response to *B. pertussis* infection to be IL-6 dependent. Decrease in antibody and cytokine production, and defect in leukocyte recruitment in the absence of IL-6, though sufficient to eliminate *B. pertussis* by day 108, appear to delay the clearance of this respiratory pathogen.

CHAPTER 2: MATERIALS AND METHODS

Bacterial strains and growth. The *B. pertussis* strain 536 used in this study, is a streptomycin resistant derivative of Tohama I [53]. It was maintained on Bordet-Gengou agar (Difco) that contained 10% defibrinated sheep blood (Hema Resources) and 20 μg/ml streptomycin. Liquid-culture bacteria were grown to mid-log phase in Stainer-Scholte [54] broth at 37°C overnight on a roller drum. Bacterial concentration was calculated according to optical density at 600 nm and bacteria were diluted to 1 x 10^7 CFU/ml in phosphate-buffered saline (PBS, OmniPur). For confirmation, dilutions of inocula were plated on Bordet-Gengou agar for colony counts.

Mice experiments. C57BL/6 (wild-type), IL-$6^{-/-}$ and Igh-$6^{-/-}$ (μMT) mice were obtained from Jackson Laboratories and bred in our *Bordetella*-free breeding rooms at Pennsylvania State University (University Park, PA). For inoculation, 4-6 week-old mice were lightly sedated with 5% Isofluorane (Abbott Laboratories) in oxygen and inoculated by pipetting 50μl of PBS containing ~5 x 10^5 CFU of bacteria onto the tip of the external nares [53, 55]. This method reliably distributes the bacteria throughout the respiratory tract [55]. For adoptive transfer of serum antibodies, 200ul of serum collected from naïve mice or convalescent mice (day 28 postinoculation), which contains *B. pertussis*-specific antibodies, was injected intraperitoneally (i.p.) at the time of inoculation [20]. For challenge experiments, mice were inoculated with 5 x 10^5 CFU of the indicated bacteria and were allowed to recover for 3 weeks. On day 21 postinoculation gentamicin was added to their drinking water (10mg/ml) for 3-4 days. On day 28 post-inoculation, mice were challenged with *B. pertussis* and dissected 3 days post challenge [56]. All protocols and animal handling were in accordance with institutional guidelines and IACUC regulations.

Bacterial Colony Forming Units (CFU) quantifications. To determine bacterial numbers in the organs, mice were sacrificed via CO_2 inhalation at the indicated timepoints. Their lungs,

tracheae, and nasal cavities were excised, homogenized in 1 ml of PBS, serial diluted and plated onto Bordet-Gengou agar containing 20μg/ml of streptomycin. Plates were incubated at 37°C for 3-4 days followed by a colony count. The lower limit of detection was 10 CFU of bacteria and is indicated by a dashed line on all appropriate graphs.

Splenocyte restimulations. Spleens were excised on the indicated day from C57BL/6 and IL-$6^{-/-}$ mice inoculated with *B. pertussis.* Splenocytes were isolated by homogenizing spleens through a wire sieve and spinning at 1500 rpm for 5 min at 4˚C [57]. Red blood cells were lysed with 0.84% ammonium chloride treatment and then the cells were washed with Dulbecco's modified Eagle cell culture medium (DMEM) (Hyclone). Cells were counted on a hemocytometer and brought to a concentration of 1 x 10^7 cells/ml in DMEM supplemented with 10% fetal calf serum (Hyclone), 1 mM sodium pyruvate (Hyclone), non-essential amino acids, and 100ug/ml penicillin and streptomycin (Hyclone). 2 x 10^6 cells were placed into each well of a 96 well tissue culture plate. The splenocytes were stimulated with medium alone or restimulated by the addition of 1 x 10^7 heat killed *B. pertussis* cells (multiplicity of infection [MOI] of 5). After 3 days of incubation, supernatants were collected and analyzed for cytokine production as described below. All samples were run in triplicates and statistical significance was determined via student's *t* -test.

Antibody and cytokine detection. For detection of *B. pertussis* specific antibodies in the serum, serum collected from C57BL/6 and IL-$6^{-/-}$ convalescent mice on day 28, 49 and 77 post-inoculation was analyzed by enzyme-linked immunosorbent assay (ELISA). In brief, 96 well plates were coated with heat-killed *B. pertussis* and were then probed with indicated convalescent-phase serum serially diluted across the plate in 1:2 ratios. Plates were probed with 1:4000 dilution of goat anti-mouse Ig, IgG1, IgG2a, IgG2b and IgM horseradish peroxidase-

conjugated antibodies (Southern Biotechnology Associates and Pharmingen) and visualized with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt in phosphor-citrate buffer at an absorbance of 405 nm. Antibody titers were determined by using the end-point titer method where comparison was made to similarly treated naïve serum [58]. Cytokine concentrations for GM-CSF and IL-10 were determined by Bio-plex™ T_H1/T_H2 Panel which is a bead–based cytokine assay (Bio-Rad). This assay worked on the same principle of sandwich ELISAs consisting of premixed antibody conjugated-beads, and biotinylated detection antibodies. The 96 well plate was read on a Bio-plex suspension array system. IFN-γ concentrations were quantified by sandwich ELISAs using appropriate paired antibodies per the manufacturer's directions (R&D Systems).

Lung leukocyte and cytokine quantifications. Lungs were perfused with sterile PBS, harvested, and placed in 4 mL of Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum. Lungs and medium were then pressed through Cellector tissue sieves (Bellco Glass) for homogenization. Lung homogenate was laid over Histopaque 1119 (Sigma Aldrich) and centrifuged for 30 min at 3000 rpm at room temperature. The leukocyte layer was collected, and the total number of cells was determined by counting at 40X magnification on a hemocytometer. They were then analyzed by flow cytometry to address the percentage of leukocytes that were neutrophils and $CD4^+$ T cells. Cells were stained with FITC-labeled anti-mouse-Ly-6G, and anti-mouse-CD4, and the percentage of Ly-6G and CD4 positive cells was multiplied by the total number of leukocytes to calculate the total number of neutrophils and $CD4^+$ T cells respectively. For the quantification of cytokines, lung homogenates were examined by cytokine ELISAs (for IL-6), which were run in accordance with the suppliers' protocols (R&D Systems).

Statistical Analysis. Three to four mice were used per group for each experiment. The mean ± standard error (error bars) was determined for all appropriate data. Two-tailed, unpaired Student's t-tests were used to determine statistical significance between groups for comparing bacterial numbers, antibody titers or cytokine production. Differences were assigned statistical significance when the p value was ≤ 0.05.

CHAPTER 3: RESULTS

IL-6 is induced in response to *B. pertussis in vivo*.

As IL-6 has been shown to be elicited in response to various respiratory pathogens [49-52], we sought to determine whether IL-6 production is increased in response to *B. pertussis*. For this, wild-type mice were inoculated with 5 x 10^5 CFU of *B. pertussis.* IL-6 levels in the lung homogenates were measured at 0, 3, 18, 24, 72 hour (h) and on day 7 postinoculation. Compared to naïve mice (data not shown), IL-6 levels were significantly elevated in the lungs of challenged mice as early as 3 hour postinoculation, peaked around day 3, and remained high on day 7 postinoculation (Fig. 1). To determine if IL-6 induction is observed after 7 days of infection with *B. pertussis*, in a separate experiment, wild-type mice were inoculated with *B. pertussis* and sacrificed on days 0, 3, 7, 14, 28, 49, 77, and 108 postinoculation to examine IL-6 production in the lungs. Initial increase in IL-6 levels was again observed within 2 hours of inoculation with *B. pertussis* (day 0, Fig. 1). However, there was no significant difference between IL-6 levels observed on day 0 and 3 as well as day 0 and 7 postinoculation (Fig. 1). Also, IL-6 production on later days such as 14, 49, 77, or 108 postinoculation was not significantly higher as compared to IL-6 levels in naïve mice.

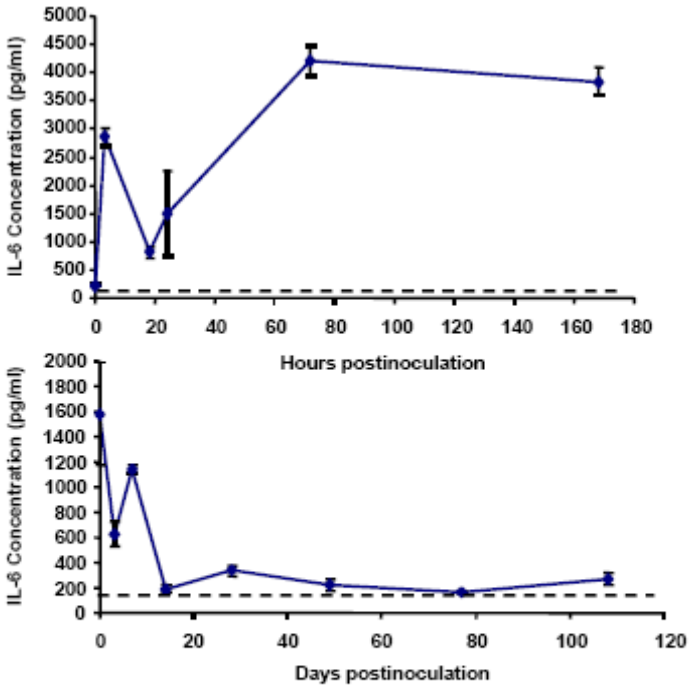

Fig. 1: IL-6 production measured in the lungs of C57BL/6 mice inoculated with *B. pertussis* and sacrificed at indicated timepoints in 2 separate experiments. IL-6 concentration is expressed as mean ± standard error (error bars). Dashed line represents IL-6 concentration for naïve C57BL/6 mice.

Combined, these data suggest that IL-6 is produced in murine lungs upon *B. pertussis* infection and its levels are highest between 3 hour to 7 days postinoculation.

IL-6 contributes to clearance of *B. pertussis* from the lower respiratory tract.

As IL-6 is induced in response to *B. pertussis* infection (Fig. 1), we sought to determine if IL-6 is important for protection against *B. pertussis*. To test this, wild-type and IL-$6^{-/-}$ mice were inoculated with 5 x 10^5 CFU of *B. pertussis* and sacrificed on days 0, 7, 14, 28, 49, 77 and 108 postinoculation for bacterial enumeration in the respiratory tract. Consistent with previous findings, *B. pertussis* load in the lungs of wild-type mice was $10^{5.82}$, $10^{6.94}$ $10^{5.26}$, $10^{3.40}$ CFU on days 0, 7, 14, 28 respectively and below limit of detection on days 49, 77, and 108 postinoculation [20]. In the lungs of IL-$6^{-/-}$ mice *B. pertussis* load was $10^{4.95}$, $10^{6.96}$, $10^{5.69}$, $10^{5.02}$, $10^{3.23}$, $10^{1.91}$, and $10^{0.95}$ on days 0, 7, 14, 28, 49, 77, and 108 postinoculation respectively (Fig. 2). The numbers of *B. pertussis* were similar in wild-type and IL-$6^{-/-}$ mice until day 14 postinoculation in

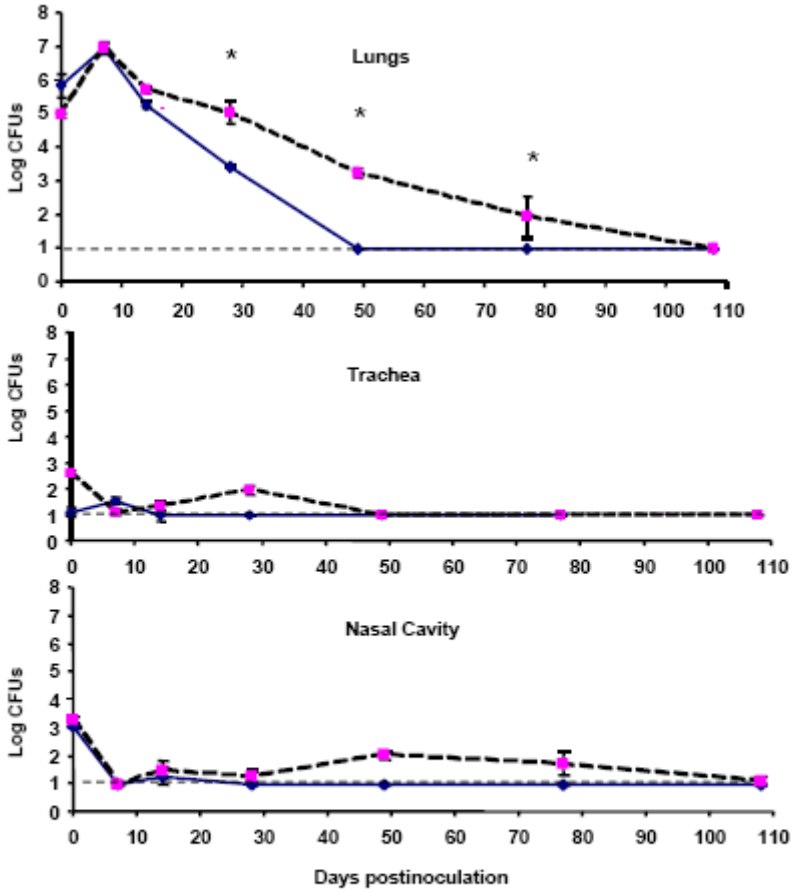

Fig. 2: *B. pertussis* load in the respiratory tract of C57BL/6 vs IL-$6^{-/-}$ mice. Groups of 3-4 C57BL/6 (solid line) and IL-6-/- (dark dashed line) mice were inoculated with *B. pertussis* and sacrificed at the indicated timepoints. Bacterial loads are expressed as the mean $\log_{10}$CFU ± standard error (error bars). The dashed line indicates the limit of detection. * indicates a *p* value ≤ 0.05

the lower respiratory tract (Fig. 2). Thereafter, *B. pertussis* colonization was about 50, 200, and 10-fold higher in the lungs of IL-6 deficient mice on days 28, 49, and 77 postinoculation respectively, compared to wild-type mice (Fig. 2). *B. pertussis* persisted for more than 11 weeks after inoculation in the lungs of IL-$6^{-/-}$ mice and was finally cleared by day 108 postinoculation. No persistence or delayed clearance was observed for *B. pertussis* in the trachea and nasal cavity of IL-$6^{-/-}$ mice (Fig. 2). IL-6 did not appear to contribute to the clearance of the two closely related pathogens, *B bronchiseptica* and *B. parapertussis* (data not shown). These data suggest that in the absence of IL-6 there is a delay in the clearance of *B. pertussis* from the lower respiratory tract.

IL-6 contributes to infection-induced immunity against *B. pertussis*.

Since there is no difference in bacterial numbers until the fourth week postinoculation (Fig. 2), it is likely that IL-6 is required for generating an effective adaptive immune response against *B. pertussis*. Therefore we hypothesized that IL-6 contributes to infection-induced immunity to *B. pertussis*. To test this, wild-type and IL-$6^{-/-}$ mice were inoculated with 5 x 10^5 CFU of *B. pertussis*. Three weeks postinoculation, mice were treated with gentamicin for 3-4 days to clear any residual *B. pertussis* [56]. Three days later, these mice, and naive wild-type and IL-$6^{-/-}$ mice were challenged with *B. pertussis* and dissected 3 days later to determine bacterial load. *B. pertussis* numbers from the convalescent wild-type mice were more than 10,000-fold lower than wild-type mice in the lungs ($10^{1.74}$ and $10^{6.27}$ CFU respectively, Fig. 3) [59]. *B. pertussis* colonization was about 30-fold lower in the lungs of convalescent IL-$6^{-/-}$ mice compared to naïve IL-$6^{-/-}$ mice ($10^{4.70}$ and $10^{6.23}$ CFU respectively). Interestingly, upon secondary challenge *B. pertussis* number was about 1000-fold higher in the lungs of IL-$6^{-/-}$ mice as compared to wild-type mice (Fig. 3). This effect of IL-6 was not seen in trachea as *B.*

pertussis load had almost cleared from both convalescent wild-type and IL-6$^{-/-}$ mice (Fig. 3). No difference was observed in bacterial numbers in the nasal cavities of any of the groups. Combined, these data suggest that IL-6 contributes to infection induced immunity to *B. pertussis* in the lower respiratory tract.

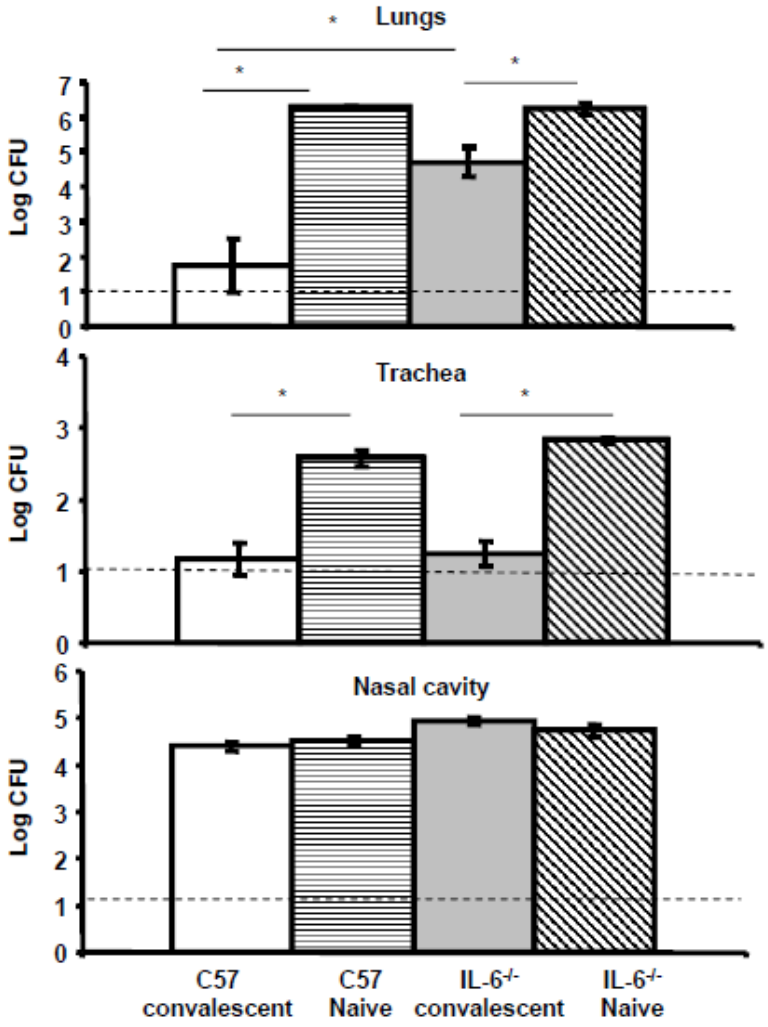

Fig. 3: *B. pertussis* load in challenged naïve and convalescent C57BL/6 and IL-6$^{-/-}$ mice. Groups of naïve or convalescent C57BL/6 or IL-6$^{-/-}$ mice were challenged with *B. pertussis* and sacrificed on day 3 post challenge. Bacterial loads are expressed as mean $\log_{10}$CFU ± standard error (error bars). The dashed line indicates the limit of detection. * indicates a p value ≤ 0.05.

IL-6 contributes to antibody production after *B. pertussis* infection.

Since IL-6 contributes to efficient infection induced immunity against *B. pertussis*, we hypothesized that this could be due to IL-6 aiding antibody production. Here, we test whether IL-6 contributes to antibody production after *B. pertussis* infection by inoculating wild-type and IL-6$^{-/-}$ mice with *B. pertussis* and sacrificing them on days 28, 49, 77 and 108 postinoculation to determine the level of *B. pertussis* specific antibody production. We quantified titers of *B. pertussis* specific polyclonal antibodies, immunoglobulin (Ig) G1, IgG2a, IgG2b and IgM by

ELISAs (Fig. 4). Wild type mice produced $10^{3.7}$, $10^{3.5}$, $10^{3.9}$, and 10^{4} titers of *B. pertussis* specific antibodies on days 28, 49, 77, and 108 respectively, the majority of which were IgG2b subtype (Fig. 4). There were approximately ten-fold lower *B. pertussis* specific antibodies in the absence of IL-6 on 28, 49, and 108 days postinoculation ($10^{2.5}$, $10^{2.8}$, and $10^{3.1}$ respectively). The level of IgG2b in serum from IL-$6^{-/-}$ mice was 10-fold lower on day 28, 49, and 108 postinoculation (Fig. 4). Combined, this data suggests that IL-6 contributes to the production of *B. pertussis* specific antibodies.

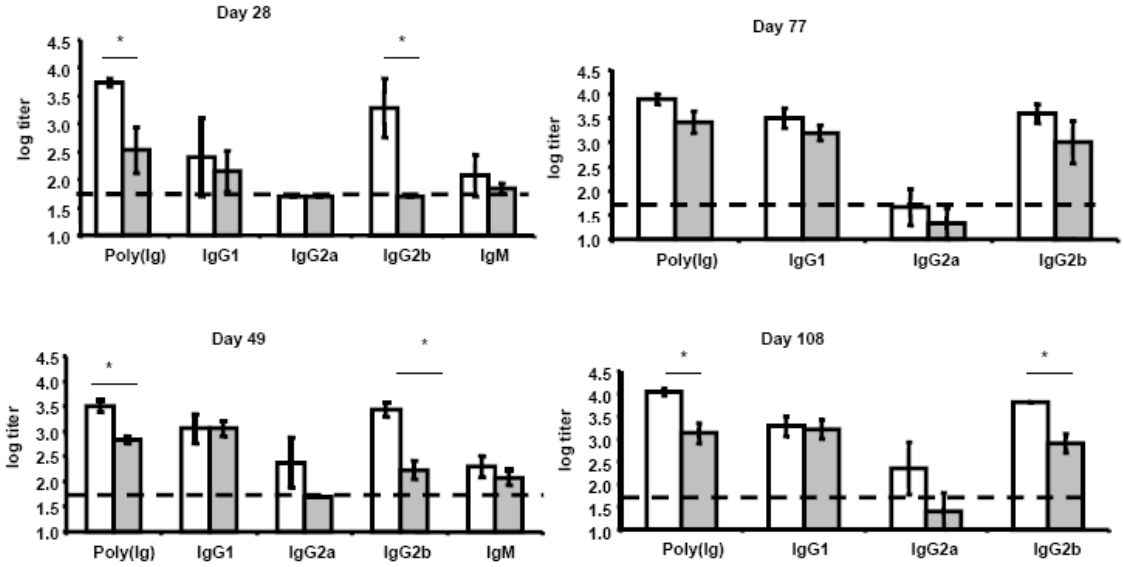

Fig. 4: Quantification of convalescent phase sera titer from wild-type and IL-$6^{-/-}$ mice. Groups of *B. pertussis* inoculated wild-type (white bar) or IL-$6^{-/-}$ (grey bar) mice were bled on the indicated day, and the resultant serum was tested for *B. pertussis* specific antibody titer. The dashed line represents the limit of detection. * indicates p value $\leq$ 0.05.

Despite lower titers, antibodies from IL-6$^{-/-}$ mice are sufficient for antibody mediated clearance of *B. pertussis*.

Next, we tested whether serum antibodies from IL-6$^{-/-}$ mice with lower *B. pertussis* specific antibody titers could lead to defective antibody mediated clearance of *B. pertussis*. We transferred immune serum from IL-6$^{-/-}$ and wild-type mice and naïve serum into μMT mice, which are deficient in antibody production and would therefore help determine if *B. pertussis* numbers are affected by antibody titer levels. At the same time we inoculated these mice with *B. pertussis*. On day 14 postinoculation, μMT mice were sacrificed, their lungs excised and bacterial load was quantified. As previously published, naïve serum was unable to reduce bacterial numbers in the lungs of μMT mice ($10^{4.91}$ CFU) [20]. *B. pertussis* numbers were about 300-fold lower in the lungs of μMT mice treated with immune sera from wild-type mice ($10^{2.44}$ CFU) compared to those treated with naïve sera (Fig 5). Interestingly, *B. pertussis* numbers were approximately $10^{2.04}$ CFU in the lungs of mice treated with immune serum from IL-6$^{-/-}$ mice even though this serum has lower *B. pertussis* specific antibody titers (Fig 5). No difference in *B. pertussis* load between mice treated with immune sera from wild-type and IL-6$^{-/-}$ mice was observed (Fig. 5). Combined, these data

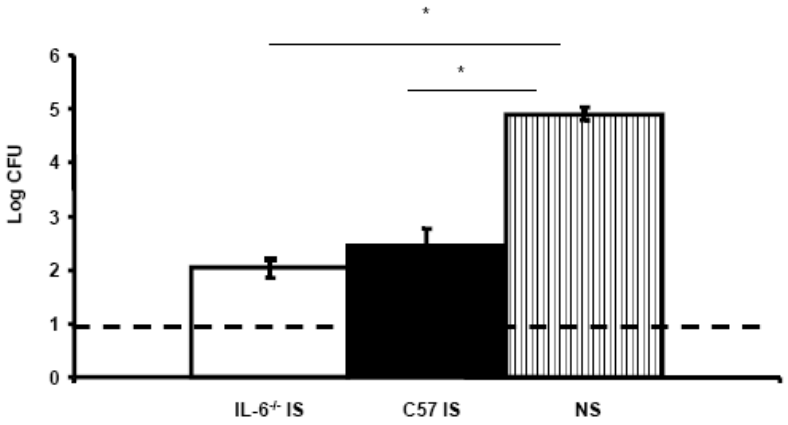

Fig. 5: The effect of immune sera from IL-6$^{-/-}$ and C57BL/6 mice on *B. pertussis* colonization in the lungs of μMT mice. Groups of 3-4 μMT mice were i.p injected with 200ul of naïve serum (NS, hatched bar), immune sera (IS) raised in wild type (closed bar), and IL-6$^{-/-}$ mice (open bar) and inoculated with *B. pertussis*. Mice were sacrificed 14 days after inoculation for bacterial enumeration in the lungs. The dashed line represents the limit of detection. The number of bacteria is expressed as the $\log_{10}$CFU mean ± standard error. * indicates $p \leq$ value 0.05.

suggest that antibodies from IL-6$^{-/-}$ mice are sufficient to decrease *B. pertussis* numbers in the lungs despite having lower antibody titers. This indicates that the defect in infection induced immunity in the absence of IL-6 is not solely caused by a decrease in antibody production in IL-6$^{-/-}$ mice.

IL-6 is not required for antibody mediated clearance of *B. pertussis*.

Based on our observation that IL-6 is required for effective infection induced immunity to *B. pertussis* (Fig. 3), we hypothesized that IL-6 may be required for the function of antibodies in immunity to *B. pertussis*. To test this, groups of wild-type and IL-6$^{-/-}$ mice were given serum collected from naive or convalescent wild-type mice by i.p. injection at the time of *B. pertussis* inoculation. These mice were sacrificed 14 days postinoculation. There were about 100 fold fewer bacteria in the lungs of wild-type mice given immune serum compared to mice which received only naïve serum ($10^{2.85}$, and $10^{4.89}$ CFU respectively, Fig. 6) [21]. Interestingly, IL-6$^{-/-}$ mice given the same immune sera were also able to lower *B. pertussis* numbers in the lungs compared to naïve serum treated IL-6$^{-/-}$ mice ($10^{2.99}$, and $10^{4.77}$ CFU respectively). Contrary to our expected result, there was no difference in antibody mediated clearance in the absence of IL-

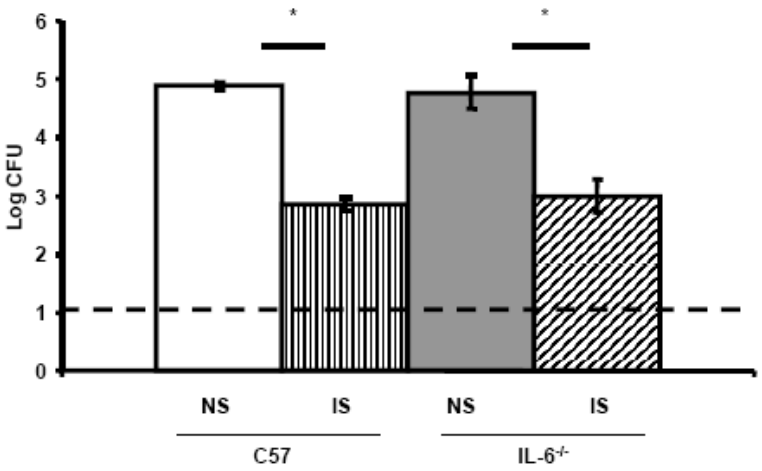

Fig. 6: Adoptively transferred antibodies lower *B. pertussis* in the lungs of wild type and IL-6$^{-/-}$ mice. Group of 3-4 C57BL/6 and IL-6$^{-/-}$ mice were injected i.p. with 200ul of naïve (NS) or immune sera (IS) and inoculated with *B. pertussis*. Mice were sacrificed 14 days after inoculation and their lungs were excised to obtain bacterial numbers. Dashed line represents limit of detection. The number of bacteria is expressed as the $\log_{10}$CFU mean ± standard error. * indicates *p* value ≤ 0.05.

6 which indicates that the function of *B. pertussis* specific antibodies on *B. pertussis* numbers is not influenced by IL-6. These data suggest that IL-6 is not required for antibody mediated clearance of *B. pertussis*.

Splenic cytokine production in IL-6 deficient mice is dampened in response to *B. pertussis*.

Lower IgG2b subtype antibody titer was observed in IL-$6^{-/-}$ mice (Fig. 4). This subtype is an indicator of T cell dependent antibody production [60]. Therefore, we hypothesized that there may be a defect in T cell mediated cytokine response upon *B. pertussis* infection in the absence of IL-6. To test this, we examined the cytokine production of splenocytes collected from wild-type and IL-$6^{-/-}$ mice 14, 28, 49, 77, and 108 days post *B. pertussis* inoculation. Production of various T_H1/T_H2 cytokines (IL-10, IL-2, IL-4, IL-5, IL-12, IFN-γ, and GM-CSF) was tested using ELISA and Bio-plex™ (Bio-Rad). IL-6 deficient splenocytes produced significantly lower GM-CSF on

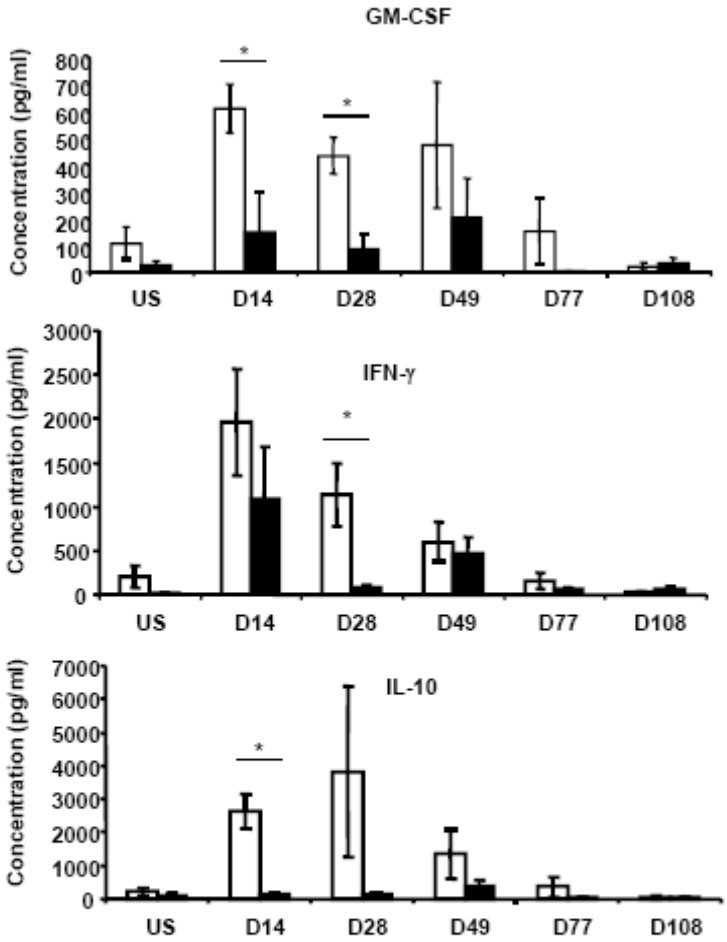

Fig. 7: Cytokine production by splenocytes from *B. pertussis* infected C57BL/6 (white bars) or IL-$6^{-/-}$ (dark bars) mice on indicated timepoints. Splenocytes were exposed to media alone (US-unstimulated), or heat-killed *B. pertussis* for 3 days. The resulting GM-CSF, IFN-γ, or IL-10 levels were measured. Concentrations are expressed as mean ± standard errors (error bars). * indicates p value ≤ 0.05.

days 14, and 28, IFN-γ on day 28, and IL-10 on day 14 postinoculation compared to splenocytes from wild-type mice (Fig. 7). Work in our lab has shown that the IFN-γ response upon *B. pertussis* infection is T cell dependent [unpublished data, Wolfe D.N.]. These data indicate that both T_H1 and T_H2 cytokine production is decreased in IL-6 deficient splenocytes upon *B. pertussis* restimulation.

Leukocyte recruitment upon *B. pertussis* infection is IL-6 dependent.

Since splenocytes from *B. pertussis* infected IL-$6^{-/-}$ mice produce significantly lower IFN-γ upon *B. pertussis* restimulation (Fig. 7), and IFN-γ has been shown to contribute to the recruitment of leukocytes during *B. pertussis* and other microbial infections [unpublished data, Wolfe D.N., 61-63], we hypothesized that IL-6 may contribute to leukocyte recruitment in the lungs in response to *B. pertussis*. To test this, groups of wild-type and IL-$6^{-/-}$ mice were inoculated with *B. pertussis* and numbers of $CD4^+$ T cells and neutrophils were quantified in the lungs 28 days postinoculation. *B. pertussis* infected wild-type mice

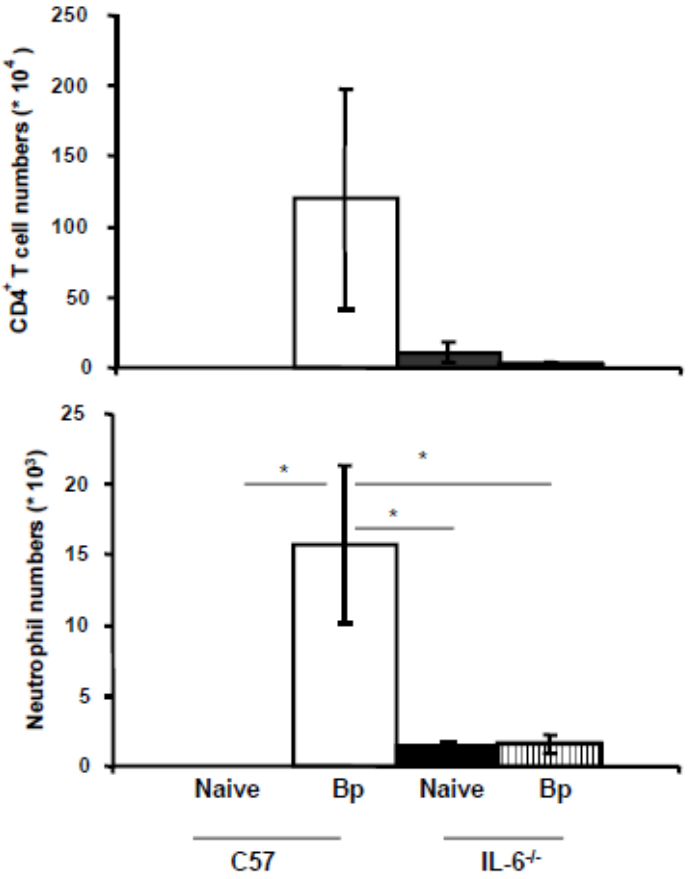

Fig. 8: Quantification of $CD4^+$T cells and neutrophils in the lungs of naïve and *B.pertussis* infected (Bp) C57BL/6 and IL-$6^{-/-}$ mice on day 28 postinoculation. Number of $CD4^+$Tcells, and neutrophils are expressed as means ± standard errors (error bars). * indicates *p* value ≤ 0.05.

showed no significant difference in numbers of $CD4^+$ T cells (~1.2 x 10^6) compared to naïve wild-type mice (~ 300 cells) but had significantly more neutrophils (~1.6 x 10^5) compared to naïve wild-type mice (no neutrophils detected) (Fig. 8). This suggests neutrophils are recruited to the lungs in response to *B. pertussis* infection. However, the number of neutrophils were same between *B. pertussis* infected and naive IL-$6^{-/-}$ mice (Fig. 8). Neutrophil (1.5 x 10^4) numbers were significantly lower in *B. pertussis* infected IL-$6^{-/-}$ mice compared to similarly treated wild-type mice (Fig. 8). These data suggest that recruitment of neutrophils to the lungs in response to *B. pertussis* infection is IL-6 dependent.

CHAPTER 4: DISCUSSION

As IL-6 plays an important role in immune responses against many microbial species, including respiratory pathogens, we were interested in investigating its role during *B. pertussis* infection. Stimulation of IL-6 production in response to *B. pertussis* (Fig. 1), lead us to study the effect of IL-6 in immunity to this reemerging pathogen. Our results show that IL-6 contributes to clearance of *B. pertussis* after two weeks of infection (Fig. 2). This effect of IL-6 was not observed for other closely related *Bordetella* species like *B. bronchiseptica* and *B. parapertussis* (data not shown) suggesting a specific role of IL-6 in *B. pertussis* clearance. IL-6 also contributes to infection-induced immunity in the lower respiratory tract against *B. pertussis* (Fig. 3). We hypothesized that this could be due to IL-6 aiding antibody production. To better understand this, we examined the production and function of *B. pertussis* specific antibodies in the absence of IL-6. While IL-6 appears to be required to generate a full antibody response to *B. pertussis* (Fig 4), it appears that this level of antibodies is sufficient to clear a *B. pertussis* infection (Fig. 5). Additionally, IL-6 does not appear to contribute to the function of *B. pertussis* specific antibodies in clearing *B. pertussis* (Fig. 6). No detectable levels of IL-6 were found in the immune serum obtained from wild type mice which was used for passive transfer into IL-$6^{-/-}$ mice (data not shown), suggesting that IL-$6^{-/-}$ mice are not lowering *B. pertussis* numbers due to any IL-6 being transferred via immune serum. Since T cells have been shown to be required for antibody mediated clearance of *B. parapertussis* [64], and *B. pertussis* [unpublished data, Wolfe D.N.], it appears that T cells are not dependent on IL-6 for antibody mediated clearance of *B. pertussis*. Together, these data suggest that while IL-6 is important in *B. pertussis* specific antibody production, it is not required for their function.

One of the antibody subtypes (IgG2b) was decreased in IL-$6^{-/-}$ mice (Fig. 3) and has previously been shown to be reduced in IL-$6^{-/-}$ mice in response to *Borrelia burgdorferi* [46].

The fact that IgG2b is known to be T cell dependent [60], indicates that T cell responses may be weaker in IL-$6^{-/-}$ mice in response to *B. pertussis* infection. To test this, we performed splenocyte restimulations with *B. pertussis* to observe systemic T cell responses upon *B. pertussis* infection and found a decrease in splenocyte cytokine response in the absence of IL-6 with decreased GM-CSF, IFN-γ, and IL-10 production (Fig. 7). Diminished IFN-γ production in the absence of IL-6 has been reported earlier during *chlamydial* infection [47]. This data suggests that there is an overall dampening of splenocyte T_H1 and T_H2 cytokine responses in the absence of IL-6. A protective role of T_H1 type immune responses against *B. pertussis* has been shown in a previous study [10]. T_H1 cytokine IFN-γ has been implicated in the intracellular killing of *B. pertussis* [65] and has been shown to contribute to the recruitment of leukocytes to the lungs upon *B. pertussis* challenge [unpublished data, Wolfe D.N.]. As IFN-γ production was decreased on day 28 postinoculation in the absence of IL-6 (Fig. 7), we hypothesized that IL-6 may contribute to leukocyte recruitment to the lungs upon *B. pertussis* infection. We observed that recruitment of leukocytes, such as neutrophils, to the lungs, in response to *B. pertussis* infection, was IL-6 dependent as accumulation of these leukocytes was observed only for infected wild type mice on day 28 postinoculation (Fig. 8). This lack of neutrophil recruitment to the site of infection is consistent with the higher *B. pertussis* numbers observed in the lungs of IL-$6^{-/-}$ mice 28 days postinoculation (Fig. 2). Together, these data suggest that delay in clearance of *B. pertussis* is likely caused by a defective T cell response in the absence of IL-6.

It has previously been shown that neutrophils, $CD4^+$ T cells, and IFN-γ are required for clearance of *B. pertussis* [21, 11, 19, unpublished data, Wolfe D.N.]. There appears to be a $CD4^+$ T cell dependent increase in IFN-γ levels during *B. pertussis* infection that increases neutrophil activation/recruitment [11, unpublished data, Wolfe D.N.]. Since IL-6 appears to be required for

efficient production of IFN-γ and recruitment of neutrophils to the lungs, it is likely important in this pathway during *B. pertussis* infection. To test this, we are currently examining if decreased IFN-γ production in IL-$6^{-/-}$ mice is T cell specific, and if neutrophil recruitment is IL-6 dependent over the entire course of *B. pertussis* infection.

IL-6 is one of the key pro-inflammatory cytokines produced by human airway epithelial cells in response to virulence factors of respiratory pathogens such as *Pseudomonas aeruginosa,* and *Haemophilus influenzae* [49-51]. IL-6 downregulates activation of cytokine network and contributes to host defense against *Streptococcus pneumoniae* infection [52]. A role of IL-6 has been established for immunity against a variety of bacterial species such as *Escherichia coli, Listeria monocytogenes, Borrelia burgdorferi*, and *Chlamydia trachomatis* [43, 45-47]. Here, we show an interesting relationship between IL-6 and the immune response against a reemerging respiratory pathogen, *B. pertussis*. Because efficient clearance and immunity to *B. pertussis* appear to be IL-6 dependent (Fig. 2 and 3), this could be an important concern for patients with Castleman's disease and rheumatoid arthritis who are undergoing the trials for new anti-IL-6 treatment [66-69]. Blockage of IL-6 and its receptor may increase *B. pertussis* persistence or susceptibility to reinfection due to defective infection-induced immunity in these patients.

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