

Epithelial Anion Transporter Pendrin Contributes to Inflammatory Lung Pathology in Mouse Models of *Bordetella pertussis* Infection

Karen M. Scanlon,^a Yael Gau,^a Jingsong Zhu,^a Ciaran Skerry,^a Susan M. Wall,^b Manoocher Soleimani,^c Nicholas H. Carbonetti^a

Department of Microbiology and Immunology, University of Maryland Medical School, Baltimore, Maryland, USA^a; Department of Medicine, Renal Division, Emory University School of Medicine, Atlanta, Georgia, USA^b; Department of Medicine, University of Cincinnati, Research Services, Veterans Affairs Medical Center, Cincinnati, Ohio, USA^c

Pertussis disease, characterized by severe and prolonged coughing episodes, can progress to a critical stage with pulmonary inflammation and death in young infants. However, there are currently no effective treatments for pertussis. We previously studied the role of pertussis toxin (PT), an important *Bordetella pertussis* virulence factor, in lung transcriptional responses to *B. pertussis* infection in mouse models. One of the genes most highly upregulated in a PT-dependent manner encodes an epithelial transporter of bicarbonate, chloride, and thiocyanate, named pendrin, that contributes to asthma pathology. In this study, we found that pendrin expression is upregulated at both gene and protein levels in the lungs of *B. pertussis*-infected mice. Pendrin upregulation is associated with PT production by the bacteria and with interleukin-17A (IL-17A) production by the host. *B. pertussis*-infected pendrin knockout (KO) mice had higher lung bacterial loads than infected pendrin-expressing mice but had significantly reduced levels of lung inflammatory pathology. However, reduced pathology did not correlate with reduced inflammatory cytokine expression. Infected pendrin KO mice had higher levels of inflammatory cytokines and chemokines than infected pendrin-expressing mice, suggesting that these inflammatory mediators are less active in the airways in the absence of pendrin. In addition, treatment of *B. pertussis*-infected mice with the carbonic anhydrase inhibitor acetazolamide reduced lung inflammatory pathology without affecting pendrin synthesis or bacterial loads. Together these data suggest that PT contributes to pertussis pathology through the upregulation of pendrin, which promotes conditions favoring inflammatory pathology. Therefore, pendrin may represent a novel therapeutic target for treatment of pertussis disease.

Bordetella pertussis, the etiologic agent of whooping cough, is a resurgent respiratory pathogen that is a major public health concern, especially for young infants (1). In 2012, almost 50,000 pertussis cases were reported in the United States, the highest number since the early 1950s, and new epidemics are emerging in various states in 2014 (<http://www.cdc.gov/pertussis/outbreaks/about.html>). Mechanisms proposed for the apparent reemergence of this disease include vaccine-driven evolution of the bacterium (2), a switch from whole-cell to acellular vaccine resulting in changes in the type and duration of immunity (3–7), and improved diagnosis (8). Severe disease, most often observed in infected infants, leads to hyperleukocytosis and can result in mortality (1, 9). However, no effective therapeutic is available to treat infants with severe pertussis.

B. pertussis modulates host innate and adaptive immune responses by releasing several toxins, including pertussis toxin (PT) (10). PT is an AB₅ toxin that binds to glycosylated molecules on target cells (11, 12), enters the cell by endocytosis, and then undergoes retrograde transport to the Golgi complex and the endoplasmic reticulum (ER), from where it enters the cytosol, likely by using the ER-associated degradation (ERAD) pathway (13–15). In the cytosol, the active subunit of PT ADP-ribosylates the α subunit of heterotrimeric G_{i/o} proteins, leading to ablated G protein-coupled receptor (GPCR) signaling, loss of inhibition of adenylyl cyclase, and increased intracellular cyclic AMP (cAMP) (16, 17). Mouse models of *B. pertussis* infection have revealed a role for active PT in enhancing colonization of the airways (18), inhibiting the protective effect of resident airway macrophages (19), inhibiting early neutrophil influx to the lungs by suppressing chemokine upregulation (20, 21), upregulating proinflammatory cytokines after the initial stages of infection (22), suppressing serum

antibody responses to *B. pertussis* (23), and exacerbating disease in various immunodeficient backgrounds (24, 25). PT is also critical for *B. pertussis*-induced infant mortality in mouse models (26), although it should be noted that the transposon insertion mutants used in that study were not complemented. However, despite our current knowledge, precisely how PT contributes to pathogenesis of pertussis remains unclear.

Through assessment of murine lung transcript levels following infection with *B. pertussis* wild-type (WT) or PT-deficient (Δ PT) strains, we previously identified a number of genes whose upregulation was associated with PT activity (27). One of the most highly upregulated genes was *slc26a4*, a gene that codes for pendrin (upregulated 9.4-fold versus phosphate-buffered saline [PBS]-treated mice and 5.4-fold versus Δ PT-infected mice). Pendrin is a transmembrane anion exchanger found on the apical surface of epithelial cells (28). This protein is highly expressed in the thyroid, kidney, and inner ear, with function-impairing mutations associated with a disease of prelingual deafness known as Pendred syndrome

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Address correspondence to Nicholas H. Carbonetti, ncarbonetti@som.umaryland.edu.

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(28–30). However, low-level *slc26a4* expression has also been described in several other organs, including the liver (31) and lungs (32). Lung expression was first identified when an allergy-driven and interleukin-13 (IL-13)-induced upregulation was observed in a murine model of asthma (32). Further studies have gone on to demonstrate that IL-13-stimulated enhanced airway *slc26a4* expression is STAT6 dependent and may also be induced by IL-4 (33, 34). In a recent publication, *slc26a4* was identified as the most highly upregulated gene in human asthmatic bronchi compared with control patients (35). In addition, levels of this anion transporter are enhanced in murine models of chronic obstructive pulmonary disease (COPD) (33) and in the sinonasal tissue of patients with allergic rhinitis and chronic rhinosinusitis (36).

In the respiratory system, pendrin functions to import Cl^- from the airway apical surface and to export both HCO_3^- and thiocyanate (SCN^-) into the airway surface liquid (ASL) (37, 38). Elevated lung pendrin levels also cause increased production of a major airway mucus protein, MUC5AC (33). In addition, SCN^- secretion supports antimicrobial mechanisms in the lung, since SCN^- is converted to the microbicidal OSCN $^-$ (hypothiocyanite) when oxidized by H_2O_2 in a reaction catalyzed by lactoperoxidase (39). Pendrin appears to contribute to airway inflammation and pathology, though the mechanism involved is unclear. In an OVA-induced model of airway hyperreactivity, pendrin knockout (KO) mice had reduced airway inflammation in response to challenge (40), while *slc26a4* overexpression caused increased mucus production and elevated production of the neutrophil-attracting chemokines CXCL1 and CXCL2 (33). However, while upregulation of *slc26a4* has been observed in response to rhinovirus infection in the presence of gamma interferon (IFN- γ) (40), to date no role for pendrin in a respiratory bacterial infection has been published.

We hypothesized that *B. pertussis*-induced elevated pendrin levels contribute to airway pathology associated with pertussis infection. In this study, we sought to examine *B. pertussis*-driven PT-dependent pendrin upregulation in the airways and to assess the contribution of this protein to respiratory inflammatory pathology in mouse models of *B. pertussis* infection.

MATERIALS AND METHODS

Bacterial strains. The *B. pertussis* strain here designated wild type (WT), is a streptomycin- and nalidixic acid-resistant derivative of Tohama I (18). The Δ PT strain, generated from the WT, does not produce PT due to an in-frame deletion spanning all 5 PT genes (18). B1834 and B1831 are *B. pertussis* clinical isolates from a Netherlands outbreak that carry the PT promoters ptxP1 and ptxP3, respectively (41). All strains were grown on Bordet-Gengou (BG) agar plates supplemented with 10% defibrinated sheep blood. WT and Δ PT growth plates also contained 200 $\mu\text{g}/\text{ml}$ streptomycin.

Mouse infections. Six- to 10-week old BALB/c (Charles River Laboratories), C57BL/6 (Charles River Laboratories or in-house bred), *Stat6* $^{-/-}$ (on the BALB/c background [42], kindly donated by A. Keegan), *Il-17a* $^{-/-}$ (on the C57BL/6 background [43], kindly donated by M. Shirliff), and *Slc26a4* $^{-/-}$ (on the C57BL/6 background [44], kindly donated by M. Soleimani) mice were all used in accordance with the University of Maryland, Baltimore (UMB), Institutional Animal Care and Use Committee. Bacterial inoculum was prepared from 48-h growth on appropriate BG agar plates in suspension with PBS. Mice were anesthetized with inhaled isoflurane, and the inoculum was administered intranasally in a 50- μl volume. Mice were inoculated with PBS, 2×10^6 CFU WT, B1831, or B1834, 1×10^7 CFU Δ PT, or 100 ng or 500 ng purified PT. Acetazolamide (100 mg/kg) was dissolved in dimethyl sulfoxide (DMSO),

diluted 1:5 in sunflower oil, and administered daily from the day of infection by subcutaneous injection. A vehicle control was prepared with DMSO and sunflower oil alone. At time of harvest, mice were euthanized by carbon dioxide inhalation, followed by thoracotomy. The lungs and trachea were divided for bacterial counts, histology, and mRNA and protein analysis. Bacterial counts were determined by serial dilution of lung homogenates that were plated onto appropriate BG agar plates and counted after 4 to 5 days of growth at 37°C. All experiments were performed at least twice, with representative data from single experiments presented.

RNA isolation and processing. Lung tissue for RNA analysis was snap-frozen in a dry ice-ethanol bath. RNA was extracted with RNA Stat60 (TelTest, Inc.) as per the manufacturer's instructions. In brief, samples were homogenized in RNA Stat60 using an Omni TH mixer (Omni, Inc.), phase separated with the addition of chloroform, and precipitated with isopropanol. RNA was quantified, and 1 μg reverse transcribed using a reverse transcription system (Promega). Quantitative real-time PCR (qRT-PCR) was performed with Maxima SYBR green/ROX quantitative PCR (qPCR) master mix (Thermo Scientific) in an Applied Biosystems 7500 Fast real-time PCR system. The hypoxanthine phosphoribosyltransferase (HPRT) gene was used as an internal housekeeping control gene (see primer details in Table S2 in the supplemental material), with all other genes normalized to the HPRT gene and expression calculated as fold change compared with PBS-inoculated control animal levels [calculated by the $2^{(-\Delta\Delta\text{CT})}$ method]. For gene analyses by RT² Profiler PCR array, RNA was isolated using a RNeasy microarray tissue minikit (Qiagen), reverse transcribed with the RT² first-strand kit (Qiagen), and profiled by the mouse innate and adaptive immune responses array (Qiagen).

Western blotting. Lung tissue for protein analysis was snap-frozen in a dry ice-ethanol bath. At the time of processing, tissue was weighed and suspended in 5 times volume HEENG buffer (20 mM HEPES [pH 7.6], 25 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10% [vol/vol] glycerol) containing 1% (vol/vol) Triton X-100, 0.05% (wt/vol) SDS, and protease inhibitor (Roche). Samples were cut, sonicated 3 times for 5-s pulses, and rotated at 4°C from 1 h. Insoluble proteins were pelleted by centrifugation at 13,000 rpm for 10 min at 4°C, and the supernatant was transferred into a new prechilled tube. Proteins were denatured in Laemmli buffer containing 15% (vol/vol) β -mercaptoethanol for 30 min at room temperature before being resolved on a 4 to 15% Mini-Protean TGX gel (Bio-Rad). Samples were transferred onto low-fluorescence polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using a Trans-Blot Turbo system (Bio-Rad). The membrane was blocked in 5% (wt/vol) milk powder formulated in Tris-buffered saline (TBS) and probed using antibodies against actin (mouse monoclonal antibody from clone AC-15; Sigma) and pendrin (rabbit polyclonal antibody raised against the C-terminal 29 amino acids [aa] of rat pendrin, kindly donated by S. Wall) (45). Antibodies were detected using goat anti-mouse IgG DyLight 680 (Thermo Scientific) and donkey anti-rabbit IRDye 800CW (Li-Cor Biosciences) and visualized by an Odyssey infrared imaging system (Li-Cor Biosciences). Pendrin was normalized to actin and results expressed as fold change compared with the average PBS-inoculated mouse lung levels.

ELISArray. Bronchoalveolar lavage (BAL) was performed in infected mice at 4 days postinoculation (dpi). In brief, an incision was made in the trachea, and a blunt-ended 21-gauge needle was inserted into the hole and sutured into place. The lungs were filled with 1 ml PBS containing 1 mM EDTA, and a volume of 700 to 800 μl was extracted. BAL fluid was kept at -80°C until processing. The ELISArray was performed as per the manufacturer's protocol (mouse inflammatory cytokine multianalyte ELISArray kit; Qiagen) using 50 μl undiluted BAL fluid per well.

Pathology. Left lung samples were selected for immunopathology. When lungs were harvested for histology, the lungs were intracardially perfused with PBS, and the right lung (cranial, medial, caudal, and post-caval lobes) was tied off and removed for other analyses. The left lung was instilled with 10% (wt/vol) buffered formalin (Sigma) via an incision in

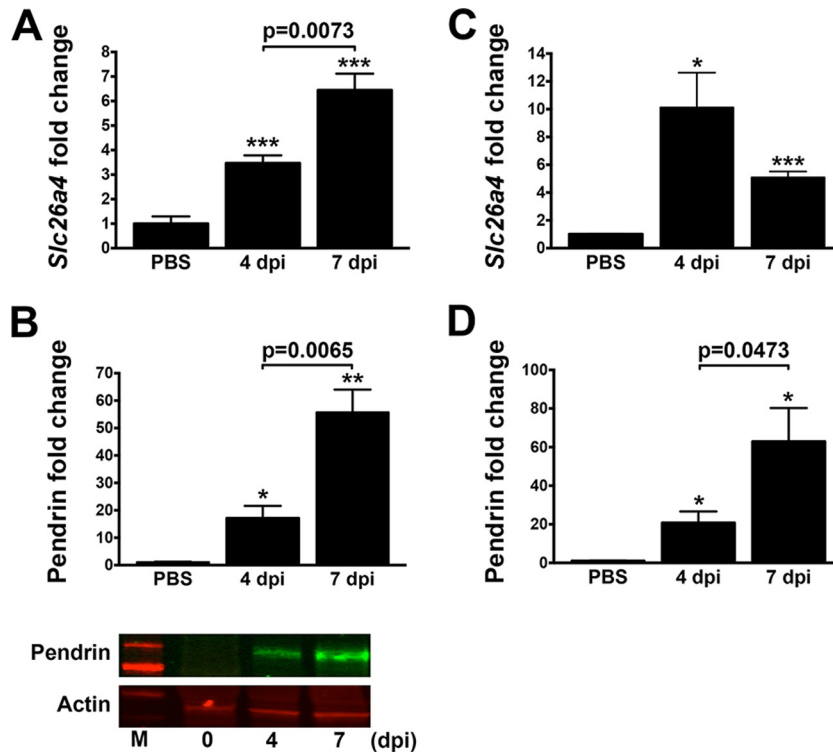


FIG 1 Pendrin production is increased upon *B. pertussis* infection. (A and B) Infected BALB/c mice ($n \geq 4$ per group) displayed upregulated *Slc26a4* expression at the mRNA level by qRT-PCR (A) and pendrin protein production as assessed by Western blotting (B) at 4 and 7 days postinfection (dpi). In a representative Western blot, the upper panel shows pendrin upregulation, the lower panel shows actin as a loading control, and M indicates the protein standard marker. (C and D) C57BL/6 mice ($n \geq 4$ per group) infected with *B. pertussis* were also evaluated at 4 and 7 dpi for *Slc26a4* transcript (C) and pendrin protein (D). Expression levels were all normalized to control PBS-treated mice and displayed as mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with control mice).

the trachea, removed, and stored in 10% (wt/vol) buffered formalin. Slides were prepared and hematoxylin and eosin (H&E) stained by the Pathology EM and Histology Laboratory (UMB core facility). Histopathology was scored on a scale of 0 to 3, with 3 being the greatest degree of pathology, for each of (i) the degree of inflammation at the site of a bronchovascular bundle (BVB), (ii) the percentage of BVB involved, and (iii) the degree of pleuritis observed, with a total possible maximum score of 9.

Statistical analysis. Graphs were plotted and data were analyzed using GraphPad Prism software. Fold changes were calculated per mouse compared with the average for the respective PBS-inoculated group. All plots represent the mean value \pm the standard error of the mean (SEM). Significance was determined by Student's *t* test for two-group analyses and by analysis of variance (ANOVA) and the Kruskal-Wallis test for experiments containing three or more groups.

RESULTS

***B. pertussis* infection in mice upregulates pendrin production in a pertussis toxin-dependent manner.** In a previous microarray study, we identified PT-dependent upregulation of the pendrin gene *slc26a4* in the lungs of *B. pertussis*-infected BALB/c mice (27). Further to this, we sought to confirm and examine this upregulation. Enhanced expression of the *slc26a4* gene and production of pendrin in lungs of infected BALB/c mice were confirmed at the mRNA (by qRT-PCR) and protein (by Western blotting) levels, respectively, compared with PBS-inoculated control animals (Fig. 1A and B). Pendrin levels were significantly enhanced at 4 days postinoculation (dpi) compared with those in PBS-treated control animals, and they further increased at 7 dpi. These results indicate that the *B. pertussis* infection causes upregulation of

slc26a4; however, whether this is through a direct or indirect mechanism is not clear. Similar *slc26a4* gene and pendrin protein upregulations were also observed in lungs of *B. pertussis*-infected C57BL/6 mice, though transcription peaked at 4 dpi (Fig. 1C and D), indicating that this function is not mouse strain specific. Enhanced *slc26a4* expression in the tracheae of infected C57BL/6 mice was not observed (data not shown), suggesting that this gene is upregulated in the bronchial and other lung tissue.

To confirm our previous findings on the role of PT in enhanced pendrin synthesis (27), we compared pendrin levels in C57BL/6 mice infected with the “wild-type” (WT) strain of *B. pertussis* to those in mice infected with a high dose of the PT-deficient strain (Δ PT). No significant difference was observed in bacterial loads between groups at 4 dpi (Fig. 2A). However, WT infection induced a significantly higher level of pendrin upregulation than Δ PT infection, supporting a role for PT in enhanced *Slc26a4* expression (Fig. 2B) ($P = 0.03$). Given that *B. pertussis* required PT for maximal pendrin expression, we tested whether treatment of mice with the toxin alone could increase expression of this protein. Mice were administered a single intranasal dose of 100 or 500 ng purified PT and probed for pendrin production in lungs by Western blotting at 4 and 7 dpi. No enhancement of pendrin protein production was observed at either time point (data not shown), indicating that PT alone is not sufficient for pendrin upregulation.

The WT strain is derived from the prototype strain Tohama, isolated in the 1950s (46). More recent *B. pertussis* strains have

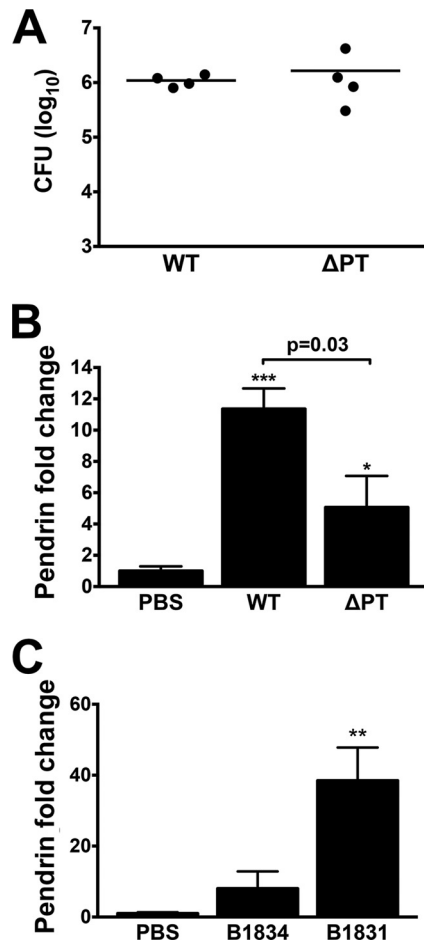


FIG 2 *B. pertussis*-induced pendrin upregulation is reduced in the absence of pertussis toxin (PT) and observed with recent clinical isolates. C57BL/6 mice ($n = 4$ per group) were infected with WT *B. pertussis* or a high dose of PT knockout *B. pertussis* (Δ PT). Lung loads were similar for both strains at 4 dpi (A). However, Δ PT-infected mice had significantly lower levels of pendrin upregulation (B). BALB/c mice ($n \geq 3$ per group) were inoculated with recent *B. pertussis* ptxP1 (B1834) or ptxP3 (B1831) clinical isolates and assessed for pendrin protein levels by Western blotting (C). Pendrin production was normalized to the control PBS-treated mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with control samples). Bars represent mean \pm SEM.

several genetic differences from Tohama, including a shift from the PT promoter P1 allele (*ptxP1*) to the P3 allele (*ptxP3*) (41). To determine whether pendrin upregulation occurs after infection with a more recent clinical *B. pertussis* isolate, we assessed pendrin protein levels in BALB/c mice infected with a ptxP1 (B1834) or ptxP3 (B1831) strain isolated in 1999 (41). Colonizations with B1834 and B1831 were at a level similar to that for the WT (data not shown); however, B1831 induced an even higher level of pendrin upregulation than that of the WT in BALB/c mice at 4 dpi (see Fig. 1B), while pendrin upregulation in B1834-infected mice was similar to that in WT-infected mice (Fig. 2C). These data show that pendrin overproduction can be induced by multiple strains of *B. pertussis* and that this is independent of the PT promoter allele.

***B. pertussis*-induced pendrin upregulation is STAT6 independent but IL-17A dependent.** Since *slc26a4* expression is upregulated by the T_H2 cytokines IL-4 and IL-13 acting through the transcription factor STAT6 in mouse models of asthma (34), we

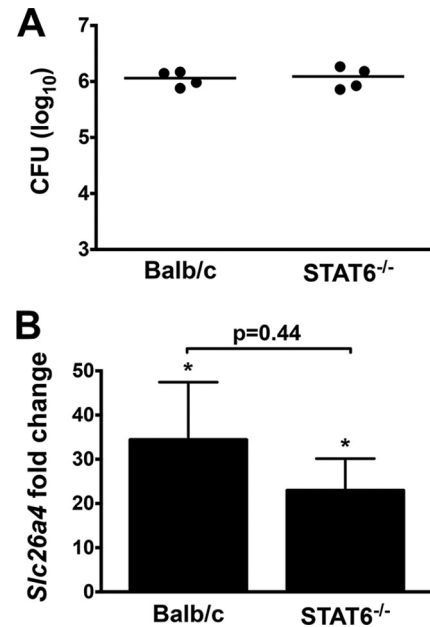


FIG 3 *B. pertussis*-induced enhanced *Slc26a4* levels are STAT6 independent. Lung loads at 4 dpi with *B. pertussis* WT were similar in BALB/c and *Stat6*^{-/-} mice ($n = 4$) (A). qPCR revealed no significant difference in infection-induced *Slc26a4* upregulation (B). Expression levels were normalized to control PBS-treated BALB/c mice and displayed as mean \pm SEM. *, $P < 0.05$ compared with control PBS-treated mice.

assessed the role of STAT6 in our model. BALB/c and STAT6 KO (*Stat6*^{-/-} on the BALB/c background) mice were inoculated with WT *B. pertussis*. At 4 dpi there was no significant STAT6-dependent difference in lung bacterial load (Fig. 3A) or *slc26a4* gene upregulation (Fig. 3B), consistent with the lack of IL-4 and IL-13 expression during *B. pertussis* infection in BALB/c mice (27). Therefore, STAT6 is not the crucial regulator of *Slc26a4* in our infection model.

In a previous study, bicarbonate secretion by human bronchial epithelial cells was significantly increased by IL-17A treatment, and it was postulated that this was due to an effect on a member of the SLC26 family of proteins (47). Since *B. pertussis* infection is associated with elevated levels of IL-17A (22, 48), we evaluated pendrin expression in infected IL-17A KO mice (on the C57BL/6 background). *Il-17a*^{-/-} mice and control C57BL/6 mice were inoculated with WT *B. pertussis* and assessed for lung bacterial load and pendrin expression at 4 dpi. No significant difference in bacterial loads was observed between the infected mouse strains (Fig. 4A). However, the level of *slc26a4*/pendrin upregulation in infected *Il-17a*^{-/-} mice was 3-fold lower than that observed in C57BL/6 mice at both the mRNA and protein levels (Fig. 4B and C), indicating that IL-17A contributes to the enhancement of *slc26a4* expression and pendrin synthesis in our model.

Pendrin KO mice exhibit reduced pertussis-driven lung pathology. Further to our finding that pendrin is upregulated in the lungs of *B. pertussis*-infected mice, we sought to determine the contribution of pendrin to pertussis infection and disease. To address this question, disease progression was assessed in infected pendrin-expressing and pendrin KO littermate mice (*Slc26a4*^{+/+} and *Slc26a4*^{-/-}, respectively). We first analyzed pendrin production in the lungs of these infected mice by Western blotting

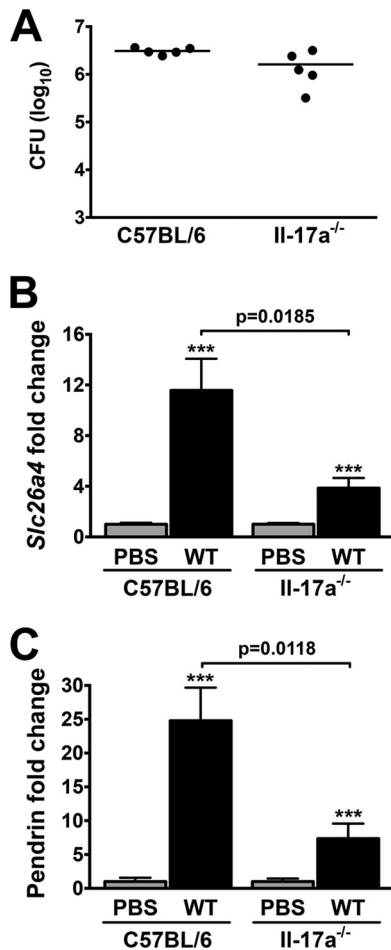


FIG 4 *B. pertussis*-induced pendrin upregulation is IL-17A dependent. Lung loads 4 dpi with WT *B. pertussis* were not significantly lower in *Il-17a*^{-/-} mice ($n = 5$) (A). However, a significant reduction in infection-induced *Slc26a4* mRNA (B) and pendrin protein (C) was observed in *Il-17a*^{-/-} mice compared with C57BL/6 mice. Expression levels were normalized to control PBS-treated samples. ***, $P < 0.001$ (compared with control strain matched mice). Bars indicate mean \pm SEM.

(Fig. 5A) and confirmed a lack of expression in the knockout mice. There was no significant difference in lung bacterial loads between mouse strains at 4 and 7 dpi, but the bacterial burden was significantly greater in *Slc26a4*^{-/-} mice at 14 and 21 dpi (Fig. 5B), demonstrating a potential antibacterial role for pendrin. However, bacterial numbers were low in both strains of mice at 28 dpi, indicating that there is no long-term deleterious effect on bacterial clearance in pendrin KO mice.

The most striking difference between infected *Slc26a4*^{+/+} and *Slc26a4*^{-/-} mice was observed in lung inflammatory pathology. *B. pertussis* infection in mice is typically characterized by peribronchial and perivascular inflammation (49). At 4 dpi, both mouse strains displayed low levels of pathology (Fig. 6E). At 7 dpi, littermates expressing pendrin exhibited peribronchiolar and perivascular lymphoid cuffing, in addition to alveolitis, slight edema, and intra-alveolar hemorrhage (Fig. 6A). In contrast, infected pendrin KO mice exhibited relatively minor bronchus-associated lymphoid tissue (BALT) hyperplasia and perivascular inflammation, in addition to a near absence of alveolitis, edema, and hemorrhage

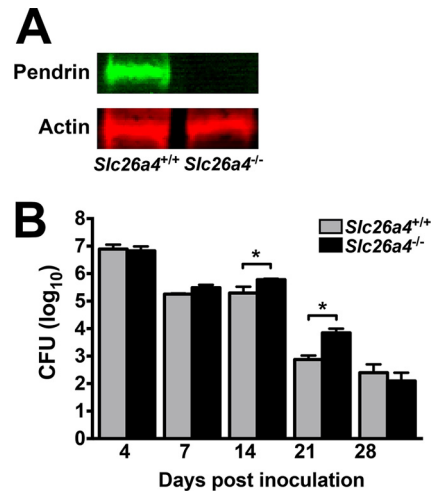


FIG 5 *B. pertussis* colonization is increased in *Slc26a4*^{-/-} mice. (A) Lack of pendrin production (upper panel) in *B. pertussis*-infected *Slc26a4*^{-/-} mice was confirmed by Western blotting, while actin levels (lower panel) remained normal. (B) Pendrin-expressing and pendrin KO littermate mice ($n \geq 5$ per group) displayed a similar course of *B. pertussis* infection. However, at later time points, lung CFU were elevated in KO mice (black bars) compared with pendrin-expressing mice (gray bars). Bars represent the average per group \pm SEM, *, $P < 0.05$.

(Fig. 6B and E). At 14 dpi, infected pendrin KO mice continued to exhibit significantly reduced pathology compared with infected pendrin-expressing littermates (Fig. 6C to E). At 21 and 28 dpi, there was continued low-level pathology in both strains that was not significantly different (Fig. 6E). We conclude that the absence of pendrin is protective against the lung inflammatory pathology induced by *B. pertussis* infection.

***B. pertussis*-infected pendrin KO mice have higher levels of inflammatory cytokines and chemokines.** *B. pertussis* infection in mice yields a T_H1/T_H17-type response associated with elevated levels of IL-17A, IFN- γ , IL-6, and tumor necrosis factor alpha (TNF- α) (22), in addition to the neutrophil- and macrophage-recruiting chemokines CXCL1, CXCL2, CXCL5, CXCL10, and CCL2 (22, 27). To determine whether the reduced lung pathology in infected pendrin KO mice was due to lower levels of proinflammatory cytokines than in infected pendrin-expressing mice, we first examined gene expression at 4 dpi with WT *B. pertussis* in *Slc26a4*^{+/+} and *Slc26a4*^{-/-} mice by PCR array. Infection in both strains of mice resulted in elevated lung expression of various proinflammatory cytokines and chemokines compared with those in PBS-treated mice (see Table S1 in the supplemental material). However, infected pendrin KO mice displayed increased levels of a number of other inflammatory mediators that were not observed in infected *Slc26a4*^{+/+} mice (see Table S1 in the supplemental material). Surprisingly, transcript levels of most of the cytokines and chemokines assayed were higher in infected *Slc26a4*^{-/-} mice than in *Slc26a4*^{+/+} mice (Fig. 7A; see Table S1 in the supplemental material). Expression of only one cytokine, IL-5, was lower in *Slc26a4*^{-/-} mice. This cytokine is typically associated with a T_H2 response and is not known to play a role in pertussis disease. To support the array data and to elaborate on the course of cytokine and chemokine expression, we assessed transcript levels at 4 and 7 dpi by qPCR for *Il-1 β* , *Il-6*, *Il-10*, *Il-17a*, *Ifn- γ* , *Cxcl1*, *Cxcl2*, *Cxcl10*, and *Ccl5*. In line with the array results, at 4 dpi most

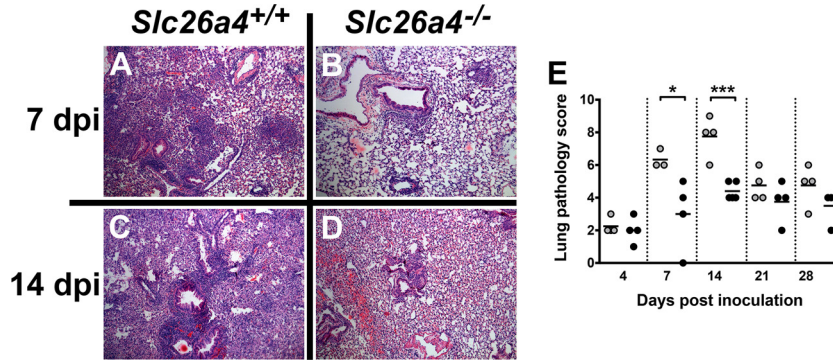


FIG 6 Lung inflammatory pathology is reduced in pendrin KO mice. A time course of *B. pertussis* infection-induced pathology was performed in pendrin-expressing (*Slc26a4*^{+/+}) and pendrin KO (*Slc26a4*^{-/-}) mice (*n* ≥ 3), and pathology was scored based on the percentage of bronchovascular bundles involved, degree of involvement, and pleuritis. (A to D) Representative H&E-stained images at 7 dpi and 14 dpi. (E) Pathology score was lower in *Slc26a4*^{-/-} mice at all time points, and this was significant at 7 dpi and 14 dpi. *, *P* < 0.05; ***, *P* < 0.001.

genes tested were upregulated to a significantly higher extent in *Slc26a4*^{-/-} than in *Slc26a4*^{+/+} mice (see Fig. S1 in the supplemental material). The only gene tested whose expression was not significantly higher in pendrin KO infected tissue was *Il-10* (see Fig.

S1 in the supplemental material), indicating that pathology is not reduced due to an increase in anti-inflammatory responses. At 7 dpi, *Il-17a* expression was still higher in pendrin KO mice, but there was little difference between mouse strains for the other cytokines and chemokines (see Fig. S1 in the supplemental material). Protein levels in bronchoalveolar lavage (BAL) fluid from infected pendrin-expressing and pendrin KO mice were assessed at 7 dpi by ELISArray. Consistent with the mRNA data, higher cytokine and chemokine protein levels were observed in *Slc26a4*^{-/-} mice than in *Slc26a4*^{+/+} mice (Fig. 7B). Taken together these data indicate that the reduced pathology in pendrin KO mice is not due to reduced production or secretion of inflammatory mediators.

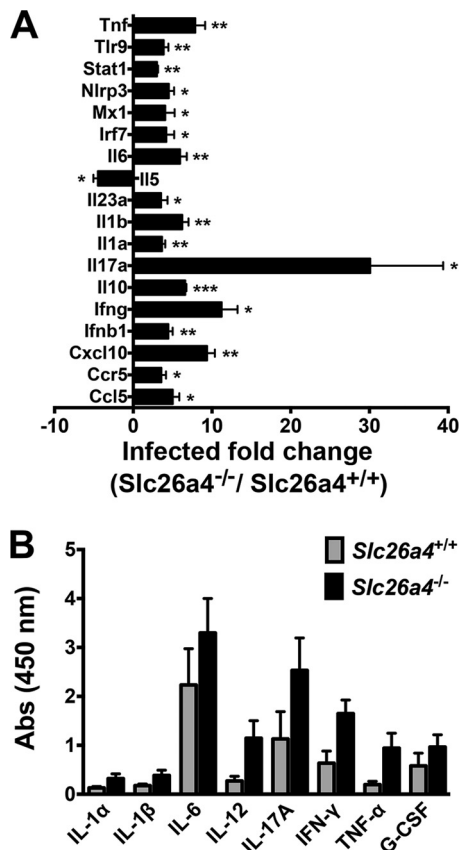


FIG 7 Cytokine and chemokine levels are higher in lungs of infected pendrin KO mice. (A) Pendrin-expressing and pendrin KO mice (*n* = 4) were infected with *B. pertussis*, and at 4 dpi lung cytokine gene mRNA levels were analyzed by RT Array (see Table S1 in the supplemental material for full data); those genes whose expression was significantly different in pendrin KO mice than in pendrin-expressing mice are displayed. (B) Protein levels for the indicated cytokines in BAL fluid were measured by ELISArray. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (relative to levels in infected pendrin-expressing mice).

Administration of a carbonic anhydrase inhibitor attenuates pertussis lung pathology. Since a dramatic reduction in *B. pertussis*-induced pathology was observed in *Slc26a4*^{-/-} mice, we next asked whether ablating bicarbonate secretion by pendrin could also attenuate *B. pertussis*-driven lung inflammatory pathology. The carbonic anhydrase inhibitor acetazolamide (ACTZ) blocks IL-17A-induced HCO₃⁻-dependent short-circuit current in human bronchial epithelial cells (47), inhibits apical Cl⁻/HCO₃⁻ exchange in type B intercalated kidney cells (50), and reduces pendrin expression in murine kidneys (31, 51) and rat kidneys (52). Therefore, we examined lung pathology in *B. pertussis*-infected mice treated daily with ACTZ. The lung bacterial burden in ACTZ-treated mice was higher than that in vehicle-treated mice, though this difference was not quite statistically significant (Fig. 8A). While ACTZ treatment did not affect airway levels of pendrin in infected mice (Fig. 8B), assessment of lung pathology revealed a significant decrease in airway inflammation in the ACTZ-treated group compared with vehicle-treated mice (Fig. 8C, F, and G). Alveolar spaces in ACTZ-treated mice had fewer inflammatory cell infiltrates, reduced inflammatory exudate, and a reduction in BAL hyperplasia. ACTZ treatment did not significantly affect cytokine or chemokine levels in infected mice (data not shown). In addition, ACTZ treatment had no significant effect on the low level of lung inflammatory pathology in pendrin KO mice (data not shown), indicating the specificity of this inhibitor for a function of pendrin. These findings with ACTZ treatment indicate that inhibition of pendrin activity results in ameliorated lung pathology associated with *B. pertussis* infection without significantly in-

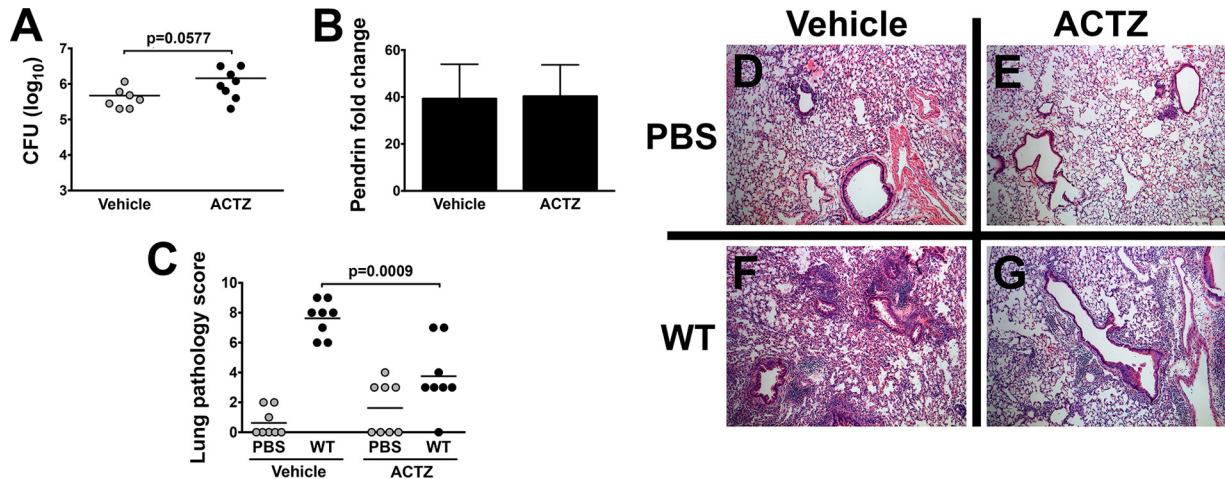


FIG 8 *B. pertussis*-induced pathology is reduced by acetazolamide (ACTZ) treatment. C57BL/6 mice ($n = 8$) were inoculated with *B. pertussis* and treated daily with vehicle or ACTZ via subcutaneous injection. (A to C) ACTZ treatment caused a slight increase in bacterial lung burden (A) and no change in pndrin protein production (B). However, lung pathology was significantly lower in ACTZ-treated mice as determined by pathology score (C). Bars represent mean \pm SEM. (D to G) Representative H&E-stained histology images.

creasing bacterial loads, suggesting a potential novel therapeutic target for pertussis.

DISCUSSION

In this study, we have shown that *B. pertussis* infection in mouse models upregulates lung expression of the epithelial anion exchanger pndrin. *Slc26a4*/pndrin was enhanced at the level of both mRNA and protein, was dependent upon PT production by the bacteria, and was independent of mouse genetic background. In addition, pndrin upregulation was not dependent on the transcription factor STAT6 but was dependent upon the cytokine IL-17A. Importantly, pndrin appears to make a significant contribution to lung pathology during *B. pertussis* infection, since pndrin KO mice suffered significantly lower levels of lung inflammatory pathology and treatment of *B. pertussis*-infected mice with the carbonic anhydrase inhibitor ACTZ also significantly reduced lung inflammatory pathology. However, inflammatory cytokine and chemokine levels were higher in pndrin KO *B. pertussis*-infected mice than in pndrin-expressing infected mice, but the mechanism of pndrin-dependent enhancement of inflammatory pathology remains to be determined.

We identified the pndrin gene as a PT-dependent upregulated gene in *B. pertussis*-infected BALB/c mouse lungs in our recently reported microarray analysis (27). In a previous microarray study of upregulated genes in *B. pertussis*-infected C3H mice, Banus et al. also found upregulation of lung *Slc26a4* expression (3.3-fold at 5 dpi versus uninfected mice) (53), and since we have observed pndrin upregulation in both BALB/c and C57BL/6 mice, this appears to be common to different genetic backgrounds. Pndrin production was apparently upregulated on the bronchial but not the tracheal epithelium in infected mice. However, preliminary immunohistochemistry analysis indicated that pndrin may also be produced by other cells in the lung tissue during *B. pertussis* infection (data not shown), although the identity of these cells and the role that their pndrin expression plays in inflammatory pathology remain to be determined.

The mechanism of pndrin gene upregulation during *B. pertussis* infection is unclear. In mouse models of asthma, pndrin

upregulation is dependent on the T_H2 cytokines IL-4 and IL-13 acting through the transcription factor STAT6 (34, 40). However, these cytokines are not produced at significant levels during *B. pertussis* infection, and our data rule out STAT6 involvement. PT is necessary for full pndrin upregulation, but it is not sufficient, indicating that other host factors associated with PT production during *B. pertussis* infection are involved. Our finding that pndrin upregulation is significantly lower in *Il-17a*^{-/-} mice suggests that IL-17A is one such host factor. These results are consistent with our previous finding that IL-17A upregulation is associated with PT production during *B. pertussis* infection (22). IL-17A was also found to induce bicarbonate secretion (a pndrin activity) in human bronchial epithelial cells (47), suggesting a role for this cytokine in pndrin upregulation in human airways. However, other host factors may also be involved, such as IL-1 β and IFN- γ , since both are upregulated during *B. pertussis* infection, are associated with PT production (27), and have been linked with pndrin upregulation in other studies (40, 54).

As well as exchanging chloride for bicarbonate ions at the lung epithelium, pndrin exports thiocyanate (SCN⁻) ions, which can be converted to microbicidal hypothiocyanite (OSCN⁻) ions (39). Therefore, pndrin upregulation may be an antimicrobial host defense mechanism at epithelial surfaces. Our observation that bacterial loads were higher in pndrin KO mice than in pndrin-expressing mice is consistent with this idea. However, our data demonstrating reduced inflammatory pathology in pndrin KO mice (despite the higher bacterial loads) and in ACTZ-treated mice strongly implicate pndrin as a contributor to lung inflammatory pathology in *B. pertussis* infection. The mechanism of pndrin-enhanced inflammatory pathology is unclear. Surprisingly, levels of inflammatory cytokines and chemokines were higher in the lungs of infected pndrin KO mice than in those of infected pndrin-expressing mice. One possibility is that pndrin increases the pH and decreases the salinity of the airway surface liquid (ASL) through its export of bicarbonate ions and import of chloride ions, respectively. These conditions may favor increased activity of antimicrobial factors, such as defensins, lysozyme, or lactoferrin, as suggested in recent study that employed a porcine model (55).

Such an environment may also favor increased activity of cytokines, chemokines, and their receptors, leading to the increased inflammatory pathology in *B. pertussis* infection. Consistent with this, Dairaghi et al. found that the activity of chemokine receptor CCR3 in its interaction with CCL11 was greatly increased by a modest increase in pH and decrease in salinity (56). In pendrin KO mice, the higher levels of cytokines and chemokines may therefore not result in inflammatory pathology because the ASL pH is correspondingly lower and the activity of these molecules is reduced. Interestingly, this situation may be similar in cystic fibrosis, where reduced function of the chloride channel CFTR may lower ASL pH and increase susceptibility to lung bacterial infection (55). Evidence exists that CFTR and SLC transporters may colocalize and interact (57) and that CFTR and pendrin may act coordinately to control ASL conditions in the lung (37, 58).

Our finding that treatment of *B. pertussis*-infected mice with ACTZ significantly reduced lung inflammatory pathology associated with the infection highlights pendrin as a possible therapeutic target for treatment of pertussis. ACTZ is a carbonic anhydrase inhibitor that is in clinical use for treatment of a variety of medical conditions, including as a respiratory stimulant in patients with COPD and metabolic alkalosis (59). Treatment from the outset of bacterial infection, as in our study, is somewhat artificial, but in future work we will investigate the therapeutic potential of this drug by treatment of infected mice at later time points of infection and by different routes of administration, since systemic inhibition of pendrin activity may lead to unwanted deleterious effects on other organs. Whether pendrin is upregulated in pertussis infection in humans remains to be determined, but it is upregulated in human asthma (35) as well as in mouse asthma models (33, 40), suggesting that the mouse model may be representative. Whether the inflammatory lung pathology seen in mouse models of pertussis is associated with the severe cough in human pertussis is unknown, but severe inflammatory pathology is a hallmark of fatal pertussis infections in young infants (9, 60). Therefore, therapeutic targeting of pendrin may be beneficial in treatment of infants with critical pertussis, for whom there are currently few, if any, effective treatments (1).

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