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Inflammasome Activation by Adenylate Cyclase Toxin Directs Th17 Responses and Protection against *Bordetella pertussis*

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Inflammasome-mediated IL-1β production is central to the innate immune defects that give rise to certain autoinflammatory diseases and may also be associated with the generation of IL-17–producing CD4+ T (Th17) cells that mediate autoimmunity. However, the role of the inflammasome in driving adaptive immunity to infection has not been addressed. In this article, we demonstrate that inflammasome-mediated IL-1β plays a critical role in promoting Ag-specific Th17 cells and in generating protective immunity against *Bordetella pertussis* infection. Using a murine respiratory challenge model, we demonstrated that the course of *B. pertussis* infection was significantly exacerbated in IL-1R type I-defective (IL-1RI−/−) mice. We found that adenylate cyclase toxin (CyaA), a key virulence factor secreted by *B. pertussis*, induced robust IL-1β production by dendritic cells through activation of caspase-1 and the NALP3-containing inflammasome complex. Using mutant toxins, we demonstrate that CyaA-mediated activation of caspase-1 was not dependent on adenylate cyclase enzyme activity but was dependent on the pore-forming capacity of CyaA. In addition, CyaA promoted the induction of Ag-specific Th17 cells in wild-type but not IL-1RI−/− mice. Furthermore, the bacterial load was enhanced in IL-17–defective mice. Our findings demonstrate that CyaA, a virulence factor from *B. pertussis*, promotes innate IL-1β production via activation of the NALP3 inflammasome and, thereby, polarizes T cell responses toward the Th17 subtype. In addition to its known role in subverting host immunity, our findings suggest that CyaA can promote IL-1β–mediated Th17 cells, which promote clearance of the bacteria from the respiratory tract. 

*B. pertussis* is the causative agent of whooping cough, a severe and debilitating respiratory infection of infants and young children, which can have a lethal outcome. Although efficacious whole-cell and acellular pertussis vaccines have been developed and are widely used, whooping cough still accounts for almost 300,000 deaths annually worldwide (1). Furthermore, there is an increasing incidence of *B. pertussis* infections in adolescents and adults, possibly due to waning immunity following the introduction of acellular vaccines into most developed countries (2). Adaptive immunity generated by previous infection or immunization with the whole-cell vaccine is effective at preventing severe disease, and studies in a mouse model suggested that a combination of Abs and Th1 and Th17 responses, and IFN-γ secreted by NK and Th1 cells seems to be critical (4, 8, 9). However, host immune responses are suppressed, especially early in infection, because of the induction of regulatory T cells (10), as well as by the various immune-subversion strategies evolved by *B. pertussis* virulence factors to prolong survival in the host (2, 11, 12).

One virulence factor of *B. pertussis*, adenylate cyclase toxin (CyaA), belongs to the repeat in toxin family of pore-forming leukotoxins (13). It is unique among the repeat in toxin family of toxins in that the characteristic cytolyisin moiety is fused to an enzymatically active adenylate cyclase (AC) enzyme domain. CyaA targets cells primarily via the integrin receptor CD11b/CD18, permeabilizes cell membranes by forming small cation-selective pores, and delivers the amino terminal AC domain into host cells (14, 15). This subverts the host immune system by catalyzing the unregulated conversion of ATP to cAMP, causing deregulated cell signaling, inhibition of bactericidal functions and potassium efflux (16), and modulation of dendritic cell (DC) maturation and cytokine secretion, reducing IL-10, while simultaneously inhibiting LPS-induced IL-12 and CCL3 production (17, 18).

NOD-like receptors (NLRs) are a family of intracellular pattern recognition receptors capable of recognizing endogenous danger signals, such as monosodium urate crystals and ATP, and exogenous substances deemed foreign, such as silica particles, alum, and asbestos (19–21). A subset of NLRs, termed NALPs, is also activated in response to bacterial pore-forming toxins. NALP1 is activated by lethal toxin from *Bacillus anthracis* (22), whereas NALP3 (NLRP3/cryopyrin) is activated by maitoxtin (23). Upon

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exposure to these toxins, a large multiprotein complex, termed the inflammasome, is formed, leading to the processing and subsequent activation of caspase-1. Active caspase-1, in turn, catalyzes the cleavage of pro–IL-1β, resulting in the secretion of biologically active forms of this cytokine (24). The precise mechanism of inflammasome activation has yet to be determined; however, it was suggested that membrane disruption, generation of reactive oxygen species, lysosomal acidification, and ionic perturbations characterized by potassium efflux may be involved.

The aim of this study was to examine the possible role of CyaA in inducing the formation of an active inflammasome complex, culminating in IL-1β production, and to assess the role of IL-1β in protective adaptive immunity to B. pertussis. We demonstrate that CyaA is capable of driving robust IL-1β production by DCs through activation of caspase-1 and the NALP3 inflammasome complex, and using a set of CyaA mutants with altered pore-forming capacity, we define the basis for this activity. Furthermore, we show that IL-1β is required for the generation of Ag-specific Th17 cells and for effective bacterial clearance, demonstrating for the first time that activation of the inflammasome is involved in protective adaptive immunity to infection.

Materials and Methods

Reagents

1,3-benzenedicarboxylic acid, 4,4′-[1,4,10,13-tetraoxa-7,16-diazacyclooctadeacene-7,16-diylbis(5-methoxy-6,2-benzofurandiyi)bis-tetrakis [acycloxy] methyl] ester (PBBF/AM) was purchased from Molecular Probes (Eugene, OR). Phor audit F-127, ATP, bafilomycin A1, and quinidine were obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure LPS was obtained from InvivoGen. Caspase-1 FLICA kit was from ImmunoChemistry Technologies (Bloomington, MN), and YVAD-fmk was from Bachem (Weil am Rhein, Germany).

Production and purification of CyaA and its derivatives

Construction of CyaA-108OV A-AC, CyaA-E509K+E516K, CyaA-E570K+15E581P, CyaA-AC, and CyaA-ΔAC was described previously (25–28). The intact CyaA and CyaA-derived proteins were produced in Escherichia coli XL-1 Blue in the presence of the activating acyltransferase CyaC and purified close to homogeneity, as previously described (29). During the hydrophobic chromatography, the resin with bound toxin was repeatedly washed with 60% isopropanol to reduce the endotoxin content of CyaA samples to <300 IU/mg protein, as determined by the Limulus ameboocyte lysate assay QCL-1000 (Cambrex, East Rutherford, NJ).

Animals and immunization

C57BL/6 (Harlan, Bicester, U.K.) and IL-1R1−/− mice, from an established colony originally from The Jackson Laboratory (Bar Harbor, ME), were bred and housed under specific pathogen-free conditions. Procedures were performed on groups of four or five mice at 8–12 wk of age according to regulations and guidelines of the Trinity College Dublin Ethics Committee and the Irish Department of Health. NALP3-deficient mice were provided by Professor Jurg Tschopp. IL-17-deficient (IL-17−/−) mice were provided by Dr. Yoichiro Ikawa (30). Mice were immunized (n = 5) s.c. in the hind foot pads with combinations of depyrogenated keyhole limpet hemocyanin (KLH) (5 µg/mouse; Calbiochem, San Diego, CA), CyaA (1 µg/mouse), YVAD-fmk (25 µg/mouse; Bachem), CyaA-E570K+E581P, or PBS only.

Cell culture

Bone marrow-derived immature DCs were prepared by culturing bone marrow cells obtained from the femur and tibia of C57BL/6 mice in RPMI 1640 and 10% FCS supplemented with 50 IU/ml GM-CSF to GM-CSF-expressing cell line, as described previously (5). Cells were washed and re-cultured with fresh medium containing 40 ng/ml GM-CSF every 3 d for 8 d. For assessment of IL-1β production, DCs were cultured (primed) with LPS (100 ng/ml) for 3 h prior to treatment for 1 h with CyaA. Supernatants were removed, after the indicated times poststimulation, and the concentrations of IL-1β, TNF-α, and IL-6 were quantified by two-site ELISA (BD Biosciences, San Jose, CA and R&D Systems, Minneapolis, MN). J774A.1 cells (ATCC TIB 67) were maintained in RPMI 1640 medium containing 10% FCS.

B. pertussis respiratory challenge and in vitro infection

B. pertussis (strain Tohama I; 338) from a 48-h culture were concentrated to 2 × 10^9/ml in PBS with 1% casein (4). Aerosol challenge was administered over 15 min using a nebulizer (0.5 ml/min). The course of B. pertussis infection was followed by performing CFU counts on lungs from groups of four mice at various times after aerosol challenge. Lungs were aseptically removed and homogenized in 1 ml sterile physiological saline with 1% casein on ice. One hundred microliters of undiluted homogenate or serially diluted homogenate from individual lungs was spotted in triplicate on Bordet-Gengou agar plates, and the number of CFU was estimated after 4 d of incubation at 37°C. Results are reported as the mean number of B. pertussis CFU for individual lungs from four mice per experimental group per time point. For in vitro infections, B. pertussis-infected wild-type (WT) strain Tohama I (338), or the WT strain 18322 when CyaA-deficient B. pertussis strain 18HS19 was used in experiments (31), at

FIGURE 1. IL-1β is required for effective clearance of B. pertussis infection. A, DCs were infected in vitro with live B. pertussis (Tohama I; 338) at an MOI of 0.1, 1, 10, or 100 for the indicated times. IL-1β concentrations were quantified by ELISA. B–D, WT and IL-1R1−/− mice were infected by aerosol challenge with B. pertussis. B, CFU were determined on individual lung homogenates. Results are expressed as mean ± SD CFU for four mice per group at each time point and are representative of two experiments. Lung (C) and spleen (D) cells were isolated from WT and IL-1R1−/− mice (n = 4) on day 14 and restimulated with heat-killed Bp (10^7/ml) or medium only. After 3 d, supernatants were tested for IL-17, IFN-γ, and IL-5 by ELISA. Results are expressed as mean ± SD and are representative of two independent experiments. **p < 0.01, ***p < 0.001; WT versus IL-1R1−/−. Bp, B. pertussis.
a multiplicity of infection (MOI) of 0.1, 1, 10, or 100. After 2, 6, or 24 h, supernatants were collected and tested for IL-1β by ELISA (R&D Systems).

Ag-specific T cell responses

Lung cells were purified as described previously (32) and restimulated with killed B. pertussis (107/ml) as prepared previously described (5). At day 14 of infection or 7 d after immunization, spleen or pleural lymph nodes were isolated and restimulated with increasing concentrations of KHL, heat-killed B. pertussis, or heat-inactivated CyaA (5 μg/ml). After 3 d, supernatants were collected and analyzed for IL-17, IFN-γ, IL-5, and IL-10 production by ELISA (BD Biosciences and R&D Systems). After 5 d, T cell cultures were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (5 μg/ml; Sigma-Aldrich) for 5 h and then were analyzed by flow cytometry.

Immunoblot analysis

Cell-culture supernatants (500 μl) were precipitated with 500 μl methanol and 250 μl chloroform prior to vortexing and centrifugation at 13,000 rpm for 5 min. The upper phase was discarded, and 500 μl methanol was added to the protein-containing interphase. This mixture was centrifuged for 5 min at 13,000 rpm, and the protein pellet was dried at 55°C. The pellet was resuspended in 60 μl Laemmli buffer prior to electrophoresis on a 15% SDS-PAGE gel. Samples were transferred to polyvinylidene difluoride membrane (Millipore) and blocked in 1% casein (Novagen, Madison, WI) prior to detection with anti–IL-1β (MAB4011; R&D Systems).

Flow cytometry

Active caspase-1 was detected using the caspase-1 FLICA kit from ImmunoChemistry Technologies. Briefly, DCs (1 × 107/ml) were treated with CyaA in the presence and absence of the unlabelled caspase-1 inhibitor YMVD-fmk for 90 min prior to treatment with the fluorescein-labeled inhibitor FAM-YMVD-fmk (5-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethyl ketone) for 1 h at 37°C. The cells were washed three times and analyzed directly by flow cytometry on a CyAN (Dako, Carpinteria, CA). T cells were stained with an Ab against CD3 (Alexa-780 conjugate) and CD4 (PE-Cy5.5 conjugate; both from eBioscience, San Diego, CA) prior to detection with anti–IL-1β (MAB4011; R&D Systems).

Fluorescence measurement of cytosolic K+

J774A.1 cells (5 × 105) grown on glass coverslips were washed in modified HBSS. Cells were loaded with 9.5 μM PBFI/AM (Molecular Probes) for 30 min at 25°C in the presence of 0.05% (w/v) Pluronic F-127 (Sigma-Aldrich) in the dark. Ratiometric measurement was performed at 25°C using the spectrofluorometer FluoroMax-3 (Jobin Yvon Horiba, Longjumeau, France) with DataMax software. Fluorescence intensity of PBFI was recorded every 15 s, and integration time for each wavelength was 3 s. Calibration experiments were performed in solution containing 50 mM HEPES (pH 7.2), with varying concentrations of potassium acetate (10, 30, 60, or 140 mM) and sodium acetate (5, 85, 115, or 135 mM). Cellular plasma membranes were permeabilized for potassium ions and protons by valinomycin and nigericin (3 μM; Sigma-Aldrich) for 30 min.

Statistical analysis

Cytokine levels were compared by one-way ANOVA. If significant differences were found, the Tukey–Kramer multiple comparisons test was used to identify differences between individual groups.

Results

IL-1 is required for effective clearance of B. pertussis infection

B. pertussis bacteria colonize the upper respiratory tract and secrete a number of virulence factors, including CyaA, which subvert host immune responses by inhibiting phagocytosis, promoting IL-10, and generating T regulatory cells, all of which contribute to bacterial persistence and pathogenesis (2). Clearance of B. pertussis from the respiratory tract is mediated by IFN-γ secreted by Th1 and NK cells, as well as Ag-specific Ab production (4, 8). Furthermore, we demonstrated that adaptive immunity induced by vaccination was dependent on Ag-specific IL-17 production (5). In this study, we examined the role of IL-1.

We first demonstrated that infection of cultured murine bone marrow-derived DCs with live B. pertussis induced robust IL-1β production in a time- and dose-dependent manner (Fig. 1A). To determine whether IL-1β plays a role in the clearance of B. pertussis infection in vivo, we challenged WT and IL-1RI−/− mice with an aerosol of B. pertussis. WT mice exhibited a classical B. pertussis infection, peaking between days 7 and 14, with successful bacterial clearance by day 35 (Fig. 1B). However, IL-1RI−/− mice exhibited a pronounced susceptibility to B. pertussis infection and had a substantially increased bacterial burden compared with WT mice at all time points following challenge. In addition, at day 35 when WT mice had successfully cleared the bacteria, IL-1RI−/− mice remained infected with bacteria, which detectable in the lungs for ≥56 d postchallenge. We next examined the cellular immune responses in the lung and spleen of infected mice on day 14; infection was associated with the induction of Ag-specific IL-17 production in WT mice, but this was significantly reduced in IL-1RI−/− mice.
mice (Fig. 1C, 1D). B. pertussis-specific IFN-γ production was also reduced in spleen and lungs of IL-1RI−/− mice, but this was not as dramatic as the reduction in IL-17 production. The Th2 cytokine IL-5 was only detected at low levels in Ag-stimulated spleen cells from B. pertussis-infected mice, and there was no significant difference between WT and IL-1RI−/− mice. These findings demonstrate a critical role for IL-1 in the generation of Ag-specific IL-17 and, to a lesser extent, IFN-γ production, as well as for successful clearance of a primary infection with B. pertussis.

CyaA induces IL-1β release by DCs

Having demonstrated that IL-1 plays a critical role in protective immunity to B. pertussis, we sought to identify the potential virulence factors involved. The processing and secretion of IL-1β by macrophages and DCs requires two signals, one resulting in the transcriptional induction of pro–IL-1β in response, for example, to a TLR agonist and a second signal culminating in cleavage and secretion of the mature cytokine through activation of the inflammasome complex. Because CyaA has pore-forming activity, we examined the possible role of this toxin in IL-1β production by inducing the formation of an active inflammasome complex. Murine bone marrow-derived DCs were treated with WT or CyaA-deficient B. pertussis for 24 h at an MOI of 1, 10, and 100, and IL-1β production was quantified by ELISA. The concentration of IL-1β induced by CyaA-deficient B. pertussis (strain:18HS19) was significantly lower than that induced by the corresponding WT B. pertussis (strain 18323) (Fig. 2A). This strain was less effective at inducing IL-1β than Tohama I, the more common strain used for the other aspects of this and other studies. Nevertheless, the data suggest that CyaA induces IL-1β production. To confirm and extend this finding, we examined the ability of purified CyaA to induce IL-1β production via inflammasome activation. The secretion of mature active IL-1β is dependent first on the induction of pro–IL-1β through TLR-induced NF-κB activation and then on the activation of caspase-1, which is required for cleavage of pro–IL-1β into mature IL-1β. Therefore, in these assays, DCs were first primed with a low concentration (100 ng/ml) of LPS for 3 h prior to treatment with increasing doses of CyaA. We found that CyaA or LPS alone did not induce IL-1β secretion. However, CyaA induced robust IL-1β production by LPS-primed DCs in a dose-dependent manner (Fig. 2B). Furthermore, Western blotting showed that CyaA promoted efficient processing of pro–IL-1β into its mature and active form to the same extent as ATP, a known inducer of IL-1β processing (Fig. 2C).

CyaA induces IL-1β production in a caspase-1–dependent manner

We next sought to determine whether CyaA drives the production of IL-1β through caspase-1, which is activated following assembly of the inflammasome complex. Murine bone marrow-derived DCs were treated with CyaA (100 ng/ml) in the presence or absence of the caspase-1 inhibitor YVAD-fmk (10 μM) or with medium only. Direct activation of caspase-1 in DCs by CyaA was assessed by flow cytometry using a fluorescein-labeled caspase-1 inhibitor. B. Pertussis DCs were treated with medium only or LPS (100 ng/ml) for 3 h before the addition of ATP (5 mM) or CyaA (100 ng/ml) in the presence or absence of the caspase-1 inhibitor YVAD-fmk (10 μM) for 1 h; IL-1β and TNF-α concentrations were measured by ELISA. Results in B and C are mean ± SE for triplicate cultures and are representative of three independent experiments.

**FIGURE 3.** CyaA activates caspase-1. A, DCs were treated with CyaA (100 ng/ml) in the presence or absence of the caspase-1 inhibitor YVAD-fmk (10 μM) or with medium only. Direct activation of caspase-1 in DCs by CyaA was assessed by flow cytometry using a fluorescein-labeled caspase-1 inhibitor. B. DCs were treated with medium only or LPS (100 ng/ml) for 3 h before the addition of ATP (5 mM) or CyaA (100 ng/ml) in the presence or absence of the caspase-1 inhibitor YVAD-fmk (10 μM) for 1 h; IL-1β and TNF-α concentrations were measured by ELISA. **FIGURE 4.** CyaA induces IL-1β production in a NALP3-dependent manner. DCs from WT or NALP3−/− mice were primed with LPS (100 ng/ml) or medium only for 3 h prior to treatment with ATP (5 mM) or CyaA (100 ng/ml) for 1 h A, IL-1β was quantified in cell supernatants by ELISA. B, Immunoblot analysis for the full-length and processed forms of IL-1β. Results are representative of two independent experiments.
The activation of caspase-1 by CyaA was assessed by flow cytometry analysis using a cell-permeable fluorescent probe that forms a covalent link with activated caspase-1. The results revealed that CyaA alone induced robust caspase-1 activation and that this was reduced in the presence of an unlabeled caspase-1 inhibitor (Fig. 3A). Pretreatment of DCs with a caspase-1 inhibitor, YVAD-fmk, significantly reduced CyaA-induced IL-1β production, but it had no effect on LPS-induced TNF-α (Fig. 3B), demonstrating a lack of off-target effects. Moreover, IL-1β production by CyaA was abrogated in DCs from caspase-1−/− mice (Fig. 3C), confirming a role for caspase-1 in CyaA-induced IL-1β.

CyaA activates the NALP3 inflammasome via pore formation and K+ efflux

Because CyaA induces pore formation and K+ release from cells (33), events that have been linked with assembly of a NALP3 containing inflammasome complex (23), we examined the hypothesis that CyaA may act through NALP3 to generate IL-1β. CyaA, like ATP, induced IL-1β secretion from LPS-primed DCs from WT, but not NALP3−/−, mice (Fig. 4A). In contrast, the induction of pro–IL-1β was similar in DCs from WT and NALP3−/− mice (Fig. 4B), confirming that the defect in IL-1β production was due to a lack of processing and caspase-1 activation. These findings demonstrate that NALP3 plays an essential role in CyaA-induced maturation of IL-1β.

We next examined the possibility that CyaA activates the NALP3 inflammasome through modulation of ionic homeostasis. IL-1β release was completely abrogated when DCs were cultured in medium containing a high concentration of potassium to prevent efflux (Fig. 5A). Similarly, pretreatment of LPS-primed DCs with quinidine, a potassium channel blocker, significantly reduced CyaA-induced IL-1β production (Fig. 5B), but it had no effect on LPS-induced IL-6 production (data not shown). CyaA can translocate directly across the cytoplasmic membrane without the need for receptor-mediated endocytosis (34). Consistent with this, we found that bafilomycin A1, an inhibitor of the vacuolar H+ ATPase, which was shown to play a role in particle-induced inflammasome activation, had no effect on CyaA-induced IL-1β release (Fig. 5B). These findings suggest that CyaA may activate the inflammasome through promotion of K+ efflux.

We next examined the role of CyaA-induced pore formation in NALP3 activation using a number of CyaA mutants with reduced or enhanced pore-forming activity (25, 28). The extent of pore formation exhibited by these mutants is reflected in their ability to induce K+ efflux and was demonstrated in macrophages loaded with a K+-sensitive probe. WT CyaA caused a rapid decrease in

**FIGURE 5.** Inflammasome activation by CyaA is dependent on pore formation and potassium efflux. A, DCs from WT mice were primed with LPS (100 ng/ml) for 2 h before being incubated in medium containing 50 mM KCl for 1 h prior to treatment with CyaA (100 ng/ml). IL-1β was quantified in cell supernatants by ELISA. ***p < 0.001; with versus without KCl. B, DCs were primed with LPS (100 ng/ml) for 2 h before being treated with quinidine (250 mM) or bafilomycin A1 (250 nM) for 1 h prior to treatment with CyaA (100 ng/ml). IL-1β was quantified in cell supernatants by ELISA. **p < 0.01; with versus without quinidine. C, J774A.1 cells were loaded with potassium-sensitive probe PBFI/AM (9.5 μM final external concentration) at 25˚C with Pluronic F-127 (12.5% [w/w]) and washed in HBSS before different CyaA mutants (3 μg/ml) or buffer control was added (arrow). Fluorescence intensity ratio of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded every 15 s. The scale on the right shows intracellular K+ values derived from calibration experiments. D, IL-1β production was assessed in DCs following treatment with WT or mutant versions of CyaA (100 ng/ml). WT CyaA = WT; E509K+E516K = enhanced pore-forming activity; E570K+E581K = reduced pore-forming activity; 108OVA-AC = no AC activity [with OVA insertion]; CyaA-ΔAC = no adenylate cyclase domain; CyaA-AC = no AC activity). Results are mean ± SE for triplicate cultures and are representative of three independent experiments.
in intracellular K⁺ (Fig. 5C). Treatment of cells with a CyaA mutant (ES09K+E516K) with enhanced pore-forming activity, but defective in AC domain translocation and cAMP accumulation, induced greater reduction in K⁺ levels (Fig. 5C), and IL-1β release was similar to WT toxin (Fig. 5D), confirming that pore formation alone is sufficient to activate NALP3. In contrast, a mutant of CyaA with defective pore-forming activity (ES70K+E851P) (13) did not affect K⁺ concentrations (Fig. 5C) and produced significantly lower amounts of IL-1β from LPS-primed DCs (Fig. 5D). The K⁺ efflux induced by CyaA is accompanied by an influx of calcium. An enzymatically inactive mutant of CyaA, which has normal pore-forming activity and K⁺ efflux, but is defective in mediating calcium influx into cells (108OV A-AC⁻) (35), induced IL-1β production similar to the WT toxin (Fig. 5D), suggesting that calcium influx was not involved. Removal of the entire AC domain in CyaA-ΔAC mutant eliminated K⁺ efflux (Fig. 5C) and abrogated the ability to induce IL-1β (Fig. 5D). Finally, mutation of the ATP-binding site within the AC domain (CyaA-AC⁻), which abolishes cAMP production but retains pore formation, was capable of reducing intracellular K⁺ concentrations (Fig. 5C) and promoting IL-1β production (Fig. 5D). Our findings demonstrate that inflammasome activation by CyaA is dependent on pore formation and K⁺ efflux but is independent of AC activity and cAMP accumulation mediated by CyaA.

**CyaA drives IL-1β-dependant Ag-specific IL-17 production in vivo**

Having shown that IL-1 plays a key role in induction of Th17 responses during *B. pertussis* infection and that CyaA promotes IL-1β production via inflammasome activation, we next sought to determine whether CyaA can promote the induction of Th17 cells in vivo. Mice were immunized with a bystander Ag, KLH, using CyaA as an adjuvant, and T cell responses were assessed. Immunization of mice with KLH alone failed to induce Ag-specific responses over that seen in mice injected with PBS alone (Fig. 6A). In contrast, lymph node cells from mice immunized with KLH and CyaA produced IL-17 in response to Ag restimulation in vitro; however, this Ag-specific IL-17 production was almost completely ablated in IL-1RI⁻/⁻ mice (Fig. 6A). CyaA did not promote Ag-specific IFN-γ production in WT or IL-1RI⁻/⁻ mice. In contrast, Ag-specific IL-10 was induced at significant levels in IL-1RI⁻/⁻ and WT mice. Further evidence that CyaA promotes the induction of Th17 cells was provided by intracellular cytokine staining on lymph node cells from immunized mice. A substantially greater frequency of IL-17-secreting CD4⁺ T cells was detected in draining lymph nodes from mice immunized with KLH and CyaA compared with those immunized with KLH alone. Furthermore, the frequency of IL-17-secreting CD4⁺CD3⁺ T cells was closer to background in similarly immunized IL-1RI⁻/⁻ mice (Fig. 6B). We next examined the role of caspase-1 activation by CyaA in mediating its ability to promote the induction of Th17 cells. Immunization of mice with KLH and CyaA in the presence of the caspase-1 inhibitor YVAD-fmk significantly reduced the induction of KLH-specific IL-17 (Fig. 7A). Furthermore, immunization with KLH in the presence of the CyaA-ES70K+E851P mutant, which is unable to form pores, failed to induce Ag-specific IL-17 production (Fig. 7A). These findings demonstrate that the promotion of Th17 cell induction by CyaA is dependent on pore formation and caspase-1 activation.

Finally, we examined the hypothesis that CyaA may also promote *B. pertussis*-specific Th17 responses. Immunization of mice with CyaA generated T cells in lymph nodes that secreted IL-17 in response to in vitro restimulation with CyaA or killed *B. pertussis*. In contrast, *B. pertussis*-specific IL-17 was not detected in IL-1RI⁻/⁻ mice (Fig. 7B). To show a direct link between IL-17 and protection from *B. pertussis*, we infected IL-17⁻/⁻ mice with *B. pertussis* and examined CFU in the lungs. The bacterial burden...
was identical after 2 h, but the CFU were significantly greater in the lungs of IL-17−/− mice compared with WT mice at 7 and 14 d after challenge (Fig. 7C). These findings demonstrate that CyaA promotes the induction of B. pertussis-specific IL-17 production and that IL-17 is required for optimum host protection against B. pertussis.

Discussion

Our findings demonstrate that IL-1β is required for effective clearance of B. pertussis infection and for the generation of Ag-specific Th17 cells, demonstrating, for the first time, a link between inflammasome activation and IL-17 production following exposure to a pathogen. We demonstrate that CyaA promotes the induction of Th17 cells, an effect that is dependent on CyaA pore formation, inflammasome activation, and IL-1β production. We also demonstrate that IL-1β plays a role in the induction of Ag-specific IFN-γ, which was previously shown to play a protective role in immunity to B. pertussis (4, 36). Although CyaA is a major virulence factor of B. pertussis and uses a variety of strategies to subvert host immune responses, our findings suggest that it may also be exploited by the host to activate protective innate and adaptive inflammatory responses against the pathogen.

Dysregulated IL-1β production through inflammasome hyper-activation is central to the pathogenesis of certain autoimmune diseases, such as Muckle-Wells syndrome (37). IL-1 also plays a central role in the pathogenesis of autoimmune disease, where it is implicated in promoting the induction or expansion of pathogenic Th17 cells (38). The present study demonstrates that IL-1β, which is produced following activation of caspase-1 and the NALP3 inflammasome by the B. pertussis virulence factor CyaA, plays a key role in the clearance of B. pertussis infection from the respiratory tract. Although it was demonstrated that NALP3 plays a crucial role in the host defense against the fungal pathogen Candida albicans (39), our study shows that inflammasome-mediated IL-1β may help to shape adaptive immune responses to infection. These findings provide evidence that innate inflammatory responses activated through the inflammasome, considered to be pathogenic in many disease settings, may also promote protective adaptive immunity to pathogens. Therefore, drugs that target IL-1β or the inflammasome pathway, as well as controlling damaging pathology in inflammatory diseases, have the potential to suppress host protective immune responses and, thus, increase susceptibility to infection.

Our findings also demonstrate that IL-1β induced by CyaA promotes the induction of Th17 cells, a population of IL-17-secreting CD4+ effector T cells distinct from the classically defined Th1/Th2 lineage that are considered to play a key pathogenic role in inflammatory diseases. Studies in mice with targeted mutations in NALP3, leading to inflammasome hyperactivation, similar to that seen in Muckle-Wells syndrome, result in excessive IL-1β production and enhanced Th17 cells (37). Although much of the focus has been on their role in promoting chronic inflammation and organ-specific autoimmunity, data are emerging to suggest that Th17 cells function with Th1 cells to mediate protective immunity against a range of pathogens. For example, a dual role for IL-17 and IFN-γ was proposed for protective immunity to Mycobacterium tuberculosis following vaccination with pathogen-derived Ags (40). IL-17 was also shown to enhance clearance of Klebsiella pneumoniae via the induction of TNF-α, IL-1β, and MIP2 production in infected lung tissue (41). We highlighted a role for IL-17 in vaccine-induced immunity to B. pertussis (5). It was also demonstrated that pertussis toxin promotes IL-17 production by T cells, macrophages, and neutrophils from B. pertussis-infected mice (7). Furthermore, neutrophils, which are induced by IL-17, were shown to play a key role in protection against B. pertussis (6, 7, 11). In the current study, we found that B. pertussis-specific IFN-γ was also reduced, although not as dramatically as IL-17 in B. pertussis-infected IL-1R1−/− mice. Consistent with our demonstration in autoimmunity (38), this suggests that IL-1 is critical for the induction of Th17 responses following infection, but it may also play a less significant role in the induction of Th1 cells. Interestingly, the bacterial burden was exacerbated in IL-17−/− mice, although not as significantly as in IL-1R1−/− mice, suggesting that some, but not all, of the effects of IL-1β involve induction of IL-17 production. These findings are consistent with the well-established role of IFN-γ (4, 36) and other effector cells and molecules, such as those induced through TLR4-activation (5, 42, 43), in protection against B. pertussis and suggest that Th1 and Th17 cells may cooperate to mediate bacterial clearance.

Consistent with previous reports on other toxins (22, 23), we found that the pore-forming activity of CyaA was essential for promoting IL-1β via activation of the inflammasome in a process that involves a reduction in intracellular potassium...
levels. Interestingly, the AC activity of CyaA, which catalyses the generation of superphysiological concentrations of intracellular cAMP and was implicated in much of the toxic and anti-inflammatory activities of CyaA (18), was not required for inactivation of pro-IL-1. It was reported that CyaA can interfere with chemotaxis, phagocytosis, and superoxide production in macrophages (16) and can enhance TLR-induced IL-10 production by DCs (17). However, these activities are dependent on AC activity and cAMP induction. Thus, although our data do not rule out a role for other activators of the inactivated required for cleavage of pro-IL-1, which is induced by LPS or other TLR agonists in B. pertussis, they do demonstrate that CyaA promotes IL-1β production, and that IL-1 is required for induction of B. pertussis-specific Th17 cells and protective immunity to B. pertussis.

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Disclosures

K.H.G.M. is a cofounder, minority shareholder, and member of the scientific advisory board of Opsome Therapeutics Ltd., a University Start-up company involved in the development of anti-inflammatory therapeutics.

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7. Amstrong, C. D., D. A. Powell, and N. H. Carbonetti. 2009. Bordetella pertussis can interfere with chemotaxis, phagocytosis, and superoxide production in macrophages (16) and can enhance TLR-induced IL-10 production by DCs (17). However, these activities are dependent on AC activity and cAMP induction. Thus, although our data do not rule out a role for other activators of the inactivated required for cleavage of pro-IL-1, which is induced by LPS or other TLR agonists in B. pertussis, they do demonstrate that CyaA promotes IL-1β production, and that IL-1 is required for induction of B. pertussis-specific Th17 cells and protective immunity to B. pertussis.

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