Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools

Nicholas H Carbonetti

Department of Microbiology & Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA ▪ Tel.: +1 410 706 7677 ▪ Fax: +1 410 706 2129 ▪ ncarbone@umaryland.edu

Abstract

Pertussis toxin and adenylate cyclase toxin are two important virulence factors of *Bordetella pertussis*, the bacterial cause of the respiratory disease pertussis or whooping cough. In addition to studies on the structure, function and role in pathogenesis of these two toxins, they are both used as cell biology tools for a variety of applications owing to their ability to enter mammalian cells, perform enzymatic activities and modify cell signaling events. In this article, recent data from the research literature that enhance our understanding of the nature of these two toxins, their role in the pathogenesis of *B. pertussis* infection and disease, particularly in modulating host immune responses, and their use as tools for other areas of research will be outlined.

Keywords

adenylate cyclase toxin; *Bordetella pertussis*; immunosuppression; pertussis toxin; respiratory infection; whooping cough

---

The disease pertussis (or whooping cough) is caused by *Bordetella pertussis*, a Gram-negative bacterial pathogen that infects the human respiratory tract. A detailed review on *B. pertussis* [1] and other useful reviews on the infection and disease have been published recently [2,3]. Unvaccinated infants and toddlers suffer the disease severest, with symptoms including paroxysmal cough with whooping and vomiting, and the pulmonary complications of the disease can be fatal in this age group [4]. Older children, adolescents and adults are also susceptible and typically suffer a persistent cough of varying severity. A whole-cell vaccine introduced in the 1940s quickly reduced the number of cases; however, owing to its reactogenicity, it has been replaced by acellular vaccines in many parts of the world over the last 20 years. However, in recent years, there has been a marked increase in the number of reported cases to a 50-year high, the cause of which is unclear but may involve reduced rates of vaccination, waning immune responses to acellular vaccines, changes in circulating strains or improved methods of surveillance and diagnosis [5].

Despite detailed molecular analysis of several virulence-associated factors of *B. pertussis*, significant gaps remain in our understanding of the pathogenesis of this infection and disease. For instance, we know next to nothing about the cause of pertussis cough or its specific

---

characteristics, and we do not know which bacterial factors contribute to the cough pathology. One of the challenges in understanding this pathology is that there are no small animal models that reproduce the specific characteristics of *B. pertussis* infection and disease in humans. However, the mouse intranasal or aerosol inoculation model has been used extensively to study respiratory tract infection by these bacteria and immune responses elicited in this host–pathogen interaction. Several characteristics of the human infection and host response are reproduced in this model, which may also be useful for the preclinical assessment of acellular pertussis vaccine efficacy [6]. In recent years, this mouse model has provided significant information on the importance and role of several virulence factors produced by the pathogen [1,3], as well as the nature of protective immune responses elicited by *B. pertussis* infection and pertussis vaccination [3,6].

Two of the most important virulence factors of *B. pertussis* are the secreted toxins pertussis toxin (PT) and adenylate cyclase toxin (ACT). The emerging picture revealed by the recent literature is that these toxins play a major role in suppression and modulation of host immune and inflammatory responses. An important question yet to be solved is whether these activities promote not only *B. pertussis* infection, but also the subsequent disease pathogenesis associated with pertussis. The topic of this article is the structure, function and activity of these toxins, their role in the pathogenesis of *B. pertussis* infection and their use as cell biology tools in other areas of research.

Pertussis toxin

Overview

Pertussis toxin is a secreted protein exotoxin and an important virulence factor produced exclusively by *B. pertussis*. PT is transported across the bacterial outer membrane by a type IV secretion system encoded by the *ptl* genes, which are located downstream from the *ptx* genes encoding the toxin [7,8]. PT is a complex multisubunit toxin with an AB5 configuration (one active subunit plus five binding subunits). The B oligomer is a pentameric ring composed of subunits S2, S3, two S4, and S5, which bind to various (but mostly unidentified) glycoconjugate molecules on the surface of target cells [9,10]. S2 and S3 each contain two different carbohydrate-binding domains [11,12], which most likely accounts for the capacity of PT to bind to and intoxicate virtually any mammalian cell (at least in culture). The enzymatic activity of PT resides in the A subunit, also known as S1. Once in the cell cytosol, S1 hydrolyzes cellular NAD and transfers the released ADP-ribose to a specific cysteine residue near the C-terminus of the α subunit of heterotrimeric G-proteins of the Gi family in mammalian cells [13]. This modification results in inhibition of Gi protein-coupled signaling pathways, causing a variety of downstream effects. PT activity requires a reducing agent to decrease the disulfide bond in S1 [14] and also requires ATP, which binds to the B oligomer to stimulate S1 dissociation from the holotoxin [15,16]. The crystal structure of PT was solved over a decade ago and revealed that S1 shares structural homology at the active site with other ADP-ribosylating bacterial toxins, and that the structure of the B oligomer is similar to that of the symmetrical B pentamer of the cholera toxin [17]. PT is known to cause most of the systemic symptoms associated with pertussis disease, such as the profound leukocytosis that may be a predictor of poor outcome in infants [18,19]. PT is an important component of acellular pertussis vaccines, and can be protective as a single-component toxoid vaccine [20]. It is also commonly used as a reagent in mammalian cell signaling studies owing to its inhibitory effect on G-protein-coupled receptor pathways.

The major issues concerning PT that have been addressed in the recent research literature are described in the following sections.

Cellular trafficking & activity

Pertussis toxin binds to glycosylated molecules on the surface of mammalian cells and enters cells by endocytosis; however, further details on the intracellular trafficking of PT to its target G-proteins in the cytosol have only recently emerged. Visualization of this trafficking by indirect immunofluorescence microscopy revealed retrograde transport of PT to the Golgi complex, but trafficking beyond this organelle was not detected by this method [21]. However, a biochemical method revealed further details on the retrograde transport pathway utilized by PT. The A (S1) or B (S4) subunits of PT were genetically tagged with peptides containing target sites for tyrosine sulfation (a Golgi-specific activity) and *N*-glycosylation (an endoplasmic reticulum [ER]-specific activity). These tagged versions of PT were found to be sulfated and then *N*-glycosylated when incubated with mammalian cells, demonstrating further retrograde transport from the Golgi complex to the ER [21]. Much of the S4 glycosylation, but not that of S1, was resistant to endoglycosidase H cleavage. This indicated that, subsequent to core *N*-glycosylation in the ER, S4 was transported anterograde to the Golgi, where further *O*-glycosylation occurred, while S1 was presumably transported to the cytosol, also suggesting that PT dissociation occurs in the ER. A mutant version of PT (PTDM) unable to bind ATP had undetectable enzymatic activity *in vitro* and in cells, but trafficked normally to the Golgi and ER. However, the *N*-glycosylation level of S1 was increased and there was no *O*-glycosylation of S4, consistent with an inability of PTDM to dissociate in the ER [Plaut R & Carbonetti N, Unpublished Data]. Furthermore, PTDM (unlike PT) could be detected in the ER by indirect immunofluorescence microscopy, supporting the role of ATP in stimulating PT dissociation in the ER and to allow further trafficking of S1. The lack of cellular activity of PTDM also indicates that the retrograde transport route is the cellular intoxication pathway for PT.

A significant question that remains unanswered is how S1 crosses the ER membrane to the cytosol. The prevailing hypothesis for PT and several other bacterial toxins that undergo retrograde transport is that they exploit the endogenous ER-associated degradation pathway that transports misfolded proteins from the ER to the cytosol for polyubiquitination and degradation by proteasomes [22]. Consistent with this hypothesis is the fact that S1 has no lysines, the primary target residue for polyubiquitination. In a recent report, engineered forms of PT with one–three lysines in place of arginines on S1 retained native *in vitro* enzymatic activity but had significantly reduced cellular activity, which could be recovered by pretreatment of cells with a proteasome inhibitor [23]. Therefore, the lack of lysines in S1 allows it to evade proteasome degradation after entry to the cytosol. Confirmation that S1 utilizes the ER-associated degradation pathway to translocate from ER to cytosol will probably depend on further molecular dissection of this pathway and the development of specific inhibitors. Trafficking of S1 within the cytosol to reach target G-proteins also requires further study. An *in vitro* study of the thermal stability of S1 showed that it is a thermally unstable protein that can be degraded in a ubiquitin-independent manner by the core 20S proteasome; however, its thermal denaturation is inhibited by interaction with NAD, the donor molecule used for ADP ribosylation of target G-proteins [24]. Thus, NAD binding may also contribute to protection of S1 from proteasome degradation in the cytosol.

Role as a virulence factor: modulation of host immune responses

Although PT is considered to be an important virulence factor of *B. pertussis*, details on its role in promoting respiratory infection and disease have only recently emerged. PT is well established as the cause of systemic symptoms associated with pertussis disease, such as leukocytosis, insuline-mia/hypoglycemia and histamine sensitivity [25], and is clearly associated with lethality of *B. pertussis* infection in a neonatal mouse model [26]. However, it was unclear from earlier studies whether PT contributed to local events of respiratory infection and disease. Several recent studies comparing wild-type and PT-deficient *B. pertussis* strains

in the young adult mouse model of infection have revealed that PT does play an important role in promoting infection in the respiratory tract, and the emerging picture is that this is achieved by suppression and modulation of various host inflammatory and immune responses to the bacteria, as described in the following sections.

Airway macrophages—Pertussis toxin-deficient mutant strains had reduced levels of airway infection in mice as soon as 24 h postinoculation [27,28], demonstrating that PT is important very early after inoculation of bacteria. Experiments with intranasal administration of purified PT showed that PT acts as a soluble factor that can enhance *B. pertussis* respiratory tract infection even when administered 14 days prior to bacterial inoculation, but not when administered just 24 h after bacterial inoculation [28]. This indicates that PT acts at an early time following bacterial inoculation and that the target cells for this PT activity are likely to be resident cells of the airways rather than newly recruited cells. One important target of this early PT activity appears to be resident airway macrophages (AMs). Depletion of AM by intranasal administration of clodronate liposomes not only enhanced infection by wild-type *B. pertussis* in mice, but also enhanced infection by the PT-deficient strain to the same high level as the wild-type strain [29]. Interestingly, intranasal treatment of mice with a single dose of PT resulted in ADP-ribosylation of AM G-proteins that lasted longer than 2 weeks (correlating with the longevity of the infection-promoting activity of PT), further supporting the conclusion that PT targets AMs to promote *B. pertussis* infection. Which specific functions of AM are inhibited by PT remains to be determined; however, there are several known PT-sensitive G-protein signaling pathways that are important in macrophage activity [30].

Neutrophils—Another early role for PT is apparently to inhibit neutrophil recruitment and influx to the airways, a common inflammatory response to bacterial infection. Using the mouse model, two groups have found that neutrophil recruitment to the lungs and airways occurs earlier after infection by a PT-deficient strain than a wild-type strain [27,28,31,32]. This was due to PT acting on resident lung cells (including AMs) to inhibit the upregulation of genes expressing chemokines (*KC*, *MIP-2* and *LIX*) that attract neutrophils, rather than a direct effect of PT on the neutrophils themselves [31]. PT may also act on pulmonary endothelial cells to inhibit neutrophil extravasation by another mechanism [33,34]. Interestingly, PT also inhibited lung chemokine gene upregulation and neutrophil recruitment in response to intranasally administered lipopolysaccharide (LPS) [31]. LPS signaling through Toll-like receptor (TLR) 4 is a major stimulus for the innate immune system in *B. pertussis* infection [35-39], and thus an important role for PT may be to inhibit this signaling. However, the PT-deficient mutant strain of *B. pertussis* still grows to lower levels than the wild-type strain in TLR4-knockout mice [Carbonetti N, Unpublished Data]. Nevertheless there are other reports from *in vitro* studies that PT can inhibit cellular responses to LPS [40,41]. The fact that TLR4 is not a G-protein-coupled receptor will make it interesting to dissect the mechanism of PT inhibition of this pathway. However, neutrophils appear to play a protective role against *B. pertussis* infection only in immune mice and in the presence of anti-*B. pertussis* antibodies. In one study, PT reduced the ability of anti-*B. pertussis* antibodies to clear infection from the lower respiratory tract of mice, an inhibitory effect that was lost after neutrophil depletion [32]. In another study, neutrophil depletion enhanced *B. pertussis* infection in immune (previously infected) mice, but not in naive mice, and did not restore full virulence to the PT-deficient strain in either mice [42]. These data indicate that neutrophils are effective against *B. pertussis* only in the presence of opsonizing antibodies, and they suggest that inhibitory effects on neutrophils are certainly not the only role for PT in *B. pertussis* infection.

Cytokine response—A very recent report on *B. pertussis* infection in the mouse model demonstrated that despite early inhibition of neutrophil recruitment by PT, this situation was quickly reversed and high numbers of neutrophils were recruited to the airways by the peak of

infection with the wild-type strain, but not with the PT-deficient strain [43]. This recruitment correlated with upregulation of neutrophil-attracting chemokine gene expression, which was due to upregulation of the cytokine IL-17. The cytokines TNF-α and IFN-γ were also upregulated at the peak of infection, indicating a mixed T-helper cell type 1 (Th1)/Th17 response. This proinflammatory cytokine upregulation was due to PT activity as well as the higher bacterial numbers in the wild-type strain infection [43]. Whether any of these cytokines play a role in clearance of the infection remains to be determined. TNF-α appears to be important since knockout mice lacking this cytokine had higher numbers of bacteria and leukocytes in their lungs than similarly infected wild-type mice and died of the infection [44]. However, a PT-deficient mutant strain was similarly attenuated in wild-type and TNF-α-knockout mice, demonstrating that this cytokine is required by the host to overcome the overall virulence effects of PT. In an *in vitro* study, IL-17 enhanced macrophage killing of *B. pertussis* [35], but in mice antibody neutralization of IL-17 only modestly increased peak bacterial loads, although it significantly reduced chemokine gene expression and neutrophil recruitment to the airways [43]. Whether PT stimulation of a proinflammatory response at the peak of infection confers an advantage to *B. pertussis* is unclear, but one possibility is that the proinflammatory response contributes to the cough pathology, promoting transmission. This hypothesis is hard to test in mice since the cough pathology is absent, and we may have to await development of a cough model in a larger animal such as a nonhuman primate.

Adaptive immune response—Pertussis toxin apparently has multiple suppressive effects on the immune system in addition to those observed on innate immune cells. Other recent studies have shown PT-mediated suppression of serum antibody responses to *B. pertussis* antigens after infection [45], reduction of MHC class II molecules on the surface of human monocytes [46] and modulation of dendritic cell surface markers [47]. Therefore, PT may promote *B. pertussis* infection by a variety of mechanisms: in the initial stages in a naive individual by inhibitory effects on innate immunity; by reducing adaptive immune responses generated by the infection; and by promoting reinfection in a partially immune individual. A role for PT in promoting pathology and transmission is also possible but is speculative at this stage.

PT in human disease—Substantial evidence has been obtained from the mouse model, but is there any direct evidence that PT promotes *B. pertussis* infection and disease in humans? PT is produced by all natural strains of *B. pertussis*, although a very recent report described a PT-deficient strain isolated from an unvaccinated infant in France who was hospitalized due to suspected pertussis [48]. However, this strain was significantly less virulent than standard strains in mouse models of infection, and it is unclear whether it was the cause of the disease in this patient. A very recent study on polymorphism in *B. pertussis* strains in The Netherlands, where a significant recent resurgence of pertussis has occurred, found an association of strains with increased PT production (due to DNA sequence changes in the *ptx* gene promoter region) with increased virulence in the human population [49]. The association of other polymorphisms in the coding sequence of the *ptx* genes for the S1 and S3 subunits with escape from vaccine immunity has also been proposed but is still speculative [5]. In addition, a wealth of data from pertussis vaccine trials has shown that detoxified PT as a single vaccine component can significantly protect humans from pertussis disease [20,50]. Final confirmation of the role for PT in human pertussis disease may have to wait for human volunteer experiments, which have not yet been approved by regulatory agencies.

B oligomer binding activity

A potential complication in understanding the role and activity of PT in any particular effect is that, in addition to the G-protein ADP-ribosylation activity of the S1 subunit, binding of the PT B oligomer (PTB) to cell surface molecules by itself can elicit several intracellular signaling

events. Some of these effects, such as lymphocyte mitogenesis, have been known for some time [10]. However, these effects typically require 10- to 100-fold higher concentrations of PT than those required for G-protein modification. PTB can be purified from the holotoxin, but its use as a reagent from this source suffers from the potential limitation that it might be contaminated with low levels of the active holotoxin, which accounts for any observed effect. Fortunately, a superior reagent is available to distinguish between PT enzymatic and binding activities, the double mutant PT9K/129G molecule developed as a vaccine component [51]. This version of PT has two amino acid substitutions in the S1 subunit that eliminate enzymatic activity, but otherwise retains normal cellular binding activity. Therefore, differences between the responses to PT and PT9K/129G can confidently be ascribed to the ADP-ribosylation activity of the S1 subunit, whereas shared responses are likely due to PT-binding activity. One of the more renowned activities of PTB is its inhibition of HIV entry and replication in T cells and macrophages, first described a decade ago [52]. In more recent studies, PTB inhibited the HIV-activating properties of the HIV Tat transactivator protein from either endogenous or extracellular sources [53], inhibited Tat-mediated induction of TGF-β in natural killer cells [54,55] and suppressed Tat-mediated apoptosis of neuronal cells [56]. PTB also inhibited IL-6-mediated induction of HIV in chronically infected promonocytic cells through its effect on the cellular transcription factor AP-1 [57]. In another study, PTB inhibited HIV infection of human cervical tissue, and the surface molecule CD14 was found to be the principal receptor for PTB in these effects [58]. Furthermore, in a surrogate animal model of HIV infection (severe combined immunodeficiency mice reconstituted with human peripheral blood leukocytes), injection of PTB strongly inhibited HIV infection and replication [59], and PTB promoted the proliferation of HIV-infected T lymphocytes from human tonsillar tissue without triggering virus replication [60]. However, whether PTB can be advanced as a potential therapeutic agent in human HIV disease remains to be determined.

Another aspect of PT activity that may be shared by PTB is adjuvanticity for certain immune responses [61]. PT and PTB may signal through TLR4 to elicit this effect, although the specifics of the response differ between the two, suggesting some role for the enzymatic activity of PT in its adjuvanticity. A recent study found that although both promoted phenotypic and functional maturation of dendritic cells, PTB induced fewer cytokine responses than PT, and PTB preferentially triggered MyD88-independent pathways while PT triggered both MyD88-dependent and -independent pathways [62]. PT9K/129G was found to signal through both TLR4 and 2, although relatively weakly through the latter, to induce proinflammatory cytokines by dendritic cells that skewed T cells towards a Th1/Th17 phenotype [63]. Another surface complex through which PTB can signal is the T-cell receptor (TCR), consistent with its mitogenic effect on T cells. Recent studies have shown that PTB triggers signaling events in T cells through the TCR in a similar manner to the commonly used T-cell stimulus anti-CD3 antibody [64], and that this signaling also causes a rapid and transient decrease in the surface expression of the chemokine receptor CXCR4 [65]. Since CXCR4 is a PT-sensitive G-protein-coupled receptor, PT possesses two activities that inhibit signaling through this receptor, short-term desensitization mediated by PTB signaling through the TCR and long-term enzymatic modification of the G-protein mediated by S1. Whether PT utilizes the TCR complex, CD14 (an LPS-binding molecule) or TLR4 as receptors for cell entry for its intoxication pathway is unclear. Another question that is still unclear is whether any of these PTB-mediated effects are relevant during *B. pertussis* infection in the respiratory tract. To address this one can compare host responses to wild-type *B. pertussis* infection with those to mutant *B. pertussis* strains that either secrete PT9K/129G (to preserve PTB effects without PT enzymatic activity) or are completely PT deficient. In our studies of the role of PT in *B. pertussis* infection and immunomodulation of the host, we have found no differences between responses to the two PT mutant strains [28,29,31,43], suggesting that PTB signaling does not contribute significantly to these aspects of the host–pathogen interaction. One reason may be that the concentration of PT produced during infection never reaches that required to elicit these PTB-

mediated effects. However, further studies will be needed to determine which of the PTB-mediated activities might be observed during *B. pertussis* infection in mice.

Role in autoimmunity

Pertussis toxin has long been known to exacerbate certain experimental autoimmune diseases in rodent models, such as experimental autoimmune encephalitis (EAE), a model of multiple sclerosis in humans [66]. However, this appears to be a complex phenomenon with multiple activities contributed by both the enzymatic activity and PTB binding effects of PT. One PT-mediated mechanism may be an increase in the permeability of the blood–brain barrier to allow pathogenic T cells access to the CNS, causing EAE pathology. In a recent study, PT, but not PTB, caused transient enhanced permeability of human brain microvascular endothelial cell monolayers and allowed elevated transmigration of human monocytes [67]. However, another study indicated that PT enhances permeability by upregulating P-selectin expression on endothelial cells to enhance leukocyte interaction with the CNS endothelium and, interestingly, that this effect was dependent on TLR4 [68]. Other studies have suggested that PT exacerbates autoimmune diseases through effects on immune cells and responses. For instance, two recent reports showed that PT reduces the number of T regulatory cells (that suppress pathogenic T-cell responses) in the spleen of EAE mice [69,70], while another study found that PT promoted the generation of a specific subset of effector T cells (CD4$^+$CD62^{Low}) that were potent in inducing autoimmunity when transferred to recipient mice [71]. In addition, PT induces IL-6 production by antigen-presenting cells and promotes the generation of Th17 cells to enhance EAE effects [72]. These findings are consistent with the emerging picture that IL-17-producing Th17 cells, rather than IFN-γ-producing Th1 cells, are the main pathogenic cells in EAE [73]. However, another study showed that PT can synergize with CNS-localized IFN-γ-induced chemokines to promote T-cell entry into the CNS and exacerbate EAE [74]. IFN-γ is also important for generating experimental autoimmune uveitis and, interestingly, recent reports have found that PT is the most potent of several TLR ligands that promote experimental autoimmune uveitis through the generation of pathogenic Th1 cells [75,76]. Overall, these observations are consistent with recent data that PT promotes the generation of a mixed Th1/Th17 response during *B. pertussis* infection [43], therefore potentially contributing to Th1- and Th17-dependent autoimmune pathologies. Whether *B. pertussis* infection itself might exacerbate these experimental autoimmune diseases has not been determined; however, the speculation exists that pertussis disease in humans may be a contributing environmental factor in widespread autoimmune diseases such as multiple sclerosis.

Adenylate cyclase toxin

Overview

Adenylate cyclase toxin is another important virulence factor secreted by *B. pertussis* (and also by other closely related *Bordetella* species). It is an immunogenic protein and can elicit a protective immune response, but it has not been included as a component of acellular pertussis vaccines. ACT consists of an amino terminal adenylate cyclase (AC) domain of approximately 400 amino acids and a pore-forming repeat in toxin (RTX) hemolysin domain of approximately 1300 amino acids with significant homology to *E. coli* hemolysin. ACT is secreted from *B. pertussis* by a type I secretion 'channel-tunnel' mechanism formed by the CyaBDE proteins, and is then modified by fatty acylation on two specific lysine residues in the hemolysin domain mediated by the CyaC acyltransferase [77]. Most of the secreted toxin appears to remain associated with the bacterial cell surface through an interaction with the bacterial adherence factor filamentous hemagglutinin, but this toxin is not active. Instead, newly synthesized and secreted ACT is the active form of the toxin [78]. Unlike PT, ACT rapidly forms inactive aggregates in solution, and so proximity of the bacteria with target host cells is necessary for ACT activity. Although ACT can intoxicate a wide variety of cells, on phagocytic cells, ACT

binds a specific surface receptor, the αMβ2 integrin or CR3 (also known as Mac-1 or CD11b/CD18) [79] through interaction with glycosyl residues on the integrin [80]. The AC domain is activated upon entry into the cell by binding to calmodulin – a mammalian cytosolic protein – to catalyze cAMP synthesis from cellular ATP. This raises the level of the key second messenger signaling molecule cAMP and disrupts cellular signaling pathways. Recently, the crystal structure of the AC domain of ACT bound to the C-terminal fragment of calmodulin was solved [81], showing an important role for a specific tryptophan residue that contacts the calcium-induced hydrophobic pocket of calmodulin, and that the calmodulin-induced conformational change of ACT is crucial for catalytic activation. Modeling of the interaction of the AC domain of ACT with the N-terminal fragment of calmodulin revealed the importance of a β-hairpin region in the AC domain and showed a very different interaction with calmodulin than another bacterial ACT, anthrax edema factor [82].

The major issues concerning ACT that have been addressed in the recent research literature are described in the following sections.

Structure & function

Acylation—Acylation of ACT is essential for its activity, but the precise role of acylation is still under investigation. Acylation enhances binding of ACT to CR3 on the surface of phagocytes [83] and acylation of residue K983 confers superior activity to that of residue K860 [84]. Acylation of K983 may also play a role in toxin structures that determine the cation selectivity of the ACT channel [84]. Oligomerization of ACT in solution, which may be required for activity, is also dependent upon acylation [85].

Pore formation & membrane translocation—Adenylate cyclase toxin forms small transient cation-selective pores in cell membranes (and lipid bilayers), which accounts for its hemolytic activity on erythrocytes; however, the relationship between pore formation and membrane translocation of the AC domain to the cell cytosol is unclear. A recent study of ACT conformations in erythrocyte membranes indicated that ACT oligomers are associated with pore formation while ACT monomers mediate AC domain translocation [86]. Each of these activities depend upon two predicted helical transmembrane domains in the pore-forming region of ACT, both of which contain crucial pairs of glutamate residues involved in these activities as well as in cation selectivity [87]. Other predicted helices in this region may also be important for ACT activity [88]. This region may also be involved in sensing voltage and pH, and therefore responding to the negative membrane potential of mammalian cells [89]. Despite these insights, much remains to be determined with regard to the mechanism of cellular entry and the relationship between pore formation and membrane translocation of ACT.

RTX domain—The RTX domain consists of glycine- and aspartate-rich nonapeptide repeats that bind calcium and are essential for ACT activity. The region may fold into a characteristic parallel β-helix motif that constitutes a novel type of calcium binding structure, as revealed by the 3D structure of related domains in other molecules [90]. Functional complementation assays and biophysical analyses revealed that the RTX functional unit includes both the nonapeptide repeats and the adjacent polypeptide segments, which are essential for the folding and calcium responsiveness of the RTX domain [91]. In addition, structural analysis showed that calcium binding induced compaction and dehydration of the RTX domain, and stabilized secondary and tertiary structures [92]. Thus, low calcium concentration in the bacterial cytosol may favor a disordered nature of the protein and facilitate its secretion through the type I machinery, while the high calcium concentration in the extracellular medium triggers folding of the RTX-containing protein into its active form.

Cellular activity

Adenylate cyclase is the primary activity of ACT affecting cell function; however, the importance of other activities of ACT and cellular effects of these activities have been revealed in the recent literature. For macrophage cytotoxicity, either pore formation or AC activity is sufficient and the two may act synergistically to enhance this effect, but ATP depletion as a result of AC activity and an unconventional calcium influx mediated by the translocating AC polypeptide may also contribute to cytotoxic effects [93-95]. ACT causes morphological changes to cells, including membrane blebbing and cell size changes to erythrocytes through its hemolytic activity, which correlates with clustering of ACT at lipid raft membrane subdomains [96]. ACT also causes cell rounding of several cultured epithelial cells due to its enzymatic activity [97]. In macrophages, the AC activity of ACT causes inactivation of the GTPase RhoA, leading to actin cytoskeleton rearrangements, membrane ruffling and loss of macropinocytic fluid phase uptake, as well as inhibition of complement-mediated phagocytosis [98]. In addition, ACT can induce apoptosis associated with caspase activation in different cells, primarily through its AC activity, and can enhance the apoptotic effects of anticancer drugs on different cancer cell lines [93,99,100].

Role as a virulence factor: modulation of host immune responses

Early work by Confer and Eaton identified ACT as a toxin secreted by *B. pertussis* that could enter cells, catalyze cAMP synthesis and inhibit phagocytosis and oxidative burst in human neutrophils [101]. Studies in mouse models established ACT as an important virulence factor for *B. pertussis* infection [26,102,103]. ACT can cause cytolysis and apoptosis of macrophages *in vitro* and *in vivo* [104-106], and ACT-deficient mutant strains of *B. pertussis* are more efficiently phagocytosed by human neutrophils [107], indicating the importance of ACT in targeting phagocytic cells for *B. pertussis* pathogenesis. In a recent study using a mouse model, the appearance of the defect in infection by an ACT-deficient mutant of *B. pertussis* occurred later than that of a PT-deficient mutant [27], consistent with data from another report showing that the ACT gene promoter was activated at a later time postinfection than the PT gene promoter [108]. The hypothesis proposed was that PT acts earlier to inhibit neutrophil influx and ACT acts later to intoxicate neutrophils (and other recruited cells) once present at the site of infection [27]. However, a more recent study found that neutrophil depletion did not enhance infection by either PT-deficient or ACT-deficient *B. pertussis* mutants in naive mice, but in neutrophil-depleted immune (previously infected) mice the ACT-deficient mutant infected at wild-type levels [42], indicating that ACT is important in neutrophil inhibition only in the presence of opsonizing antibodies.

Adenylate cyclase toxin may also have important immunomodulatory effects on the host that contribute to pathogenesis and, similar to PT, these effects seem to be a mixture of inhibitory and stimulatory activities. ACT can upregulate MHC class II and costimulatory molecules on dendritic cells, inducing a semimature state that decreases proinflammatory cytokine production [36,109,110]. This effect was due to the enzymatic activity of the toxin, while cytotoxic effects of ACT on these cells were associated with the pore-forming activity [109]. In another study, induction of IL-23, a cytokine necessary for Th17 responses, and inhibition of the Th1 cytokine IL-12 was due to the enzymatic action of ACT raising cAMP levels, since the reverse pattern of expression was observed in dendritic cells incubated with an ACT-deficient mutant of *B. pertussis*, while addition of cAMP could restore IL-23 production [111]. The enzymatic activity of ACT was also involved in promoting IL-10 production by dendritic cells by enhancing MAPK phosphorylation and inhibiting IL-12 production by suppressing TLR-induced IFN regulatory factor expression [112]. ACT also inhibited T-cell activation and chemotaxis by inhibiting TCR and chemokine receptor signaling through MAPK inhibition [113]. However, in human cells, low concentrations of ACT activated TCR signaling mediators and caused polarization of T-cell responses to a Th2 phenotype by upregulating Th2

transcription factors [114]. ACT induced cyclooxygenase-2 expression in murine macrophages through its enzymatic activity and could synergize with TLRs to achieve this effect [115]. An array study showed that ACT upregulated transcription in murine macrophages of many inflammatory genes and caused downregulation of numerous genes involved in cell proliferation, and these effects were also dependent upon its enzymatic activity [116]. Most of these are *in vitro* studies, however, and so which of these activities are relevant to *B. pertussis* infection and pathogenesis remains to be determined.

Use as an antigen delivery tool

Since ACT can target antigen-presenting cells and deliver its catalytic domain directly into the cytosol, enzymatically inactive versions of ACT have been used as delivery vectors for internalization of immunogenic epitopes. These epitopes are processed and presented by MHC class I and II molecules and stimulate CD4 and CD8 T-cell responses *in vitro* and *in vivo* that can protect against infections or tumors [117,118]. Most fusion constructs have been engineered genetically; however, chemical fusion of epitopes to ACT also achieved the same effect [119]. In recent studies, this application was used to generate an HIV Tat–ACT fusion that could elicit Tat-specific antibodies and CD8 T cells in immunized mice [120,121], and a fusion with a malarial parasite epitope that could generate parasite-specific CD8 cells and completely protect mice when used as part of a heterologous prime/boost immunization regimen [122]. Interestingly, this technology was also recently used to fuse *Mycobacterium tuberculosis* antigens to ACT, and these fusion molecules were able to restimulate IFN-γ production by T cells from TB patients, which could facilitate detection of latent infection from patient blood samples, including those from HIV-infected individuals [123,124]. Thus, this use of ACT as a delivery vector holds promise for many prophylactic, therapeutic and diagnostic applications and it remains to be seen whether this can be extended to human trials.

Use as a tool for intracellular protein detection & for protein–protein interaction

Adenylate cyclase toxin has also been used as a cell biology tool for other applications [125]. One of these is as a selective reporter for protein translocation from bacteria to eukaryotic cells, since the AC domain can be fused to other proteins and is only active intracellularly upon binding to calmodulin. In particular, this has been used to study protein targeting by type III secretion machinery in several bacteria. Examples of this from the recent literature include identification of type III-secreted effector proteins of *Salmonella enterica* serovar Typhimurium [126], type III-secreted proteins of *Yersinia pestis* that are putatively insecticidal [127], type III-secreted effectors of the plant pathogen *Xanthomonas oryzae* [128] and development of a colorimetric assay of cell viability to analyze type III secretion [129]. Another use of ACT is in genetic screens for protein–protein interaction in *E. coli*. This takes advantage of the finding that the catalytic domain has a modular structure consisting of two complementary fragments that are both required for enzymatic activity, and so each can be fused onto separate proteins to investigate their interaction, with the resulting AC activity complementing a cyclase-deficient *E. coli* strain [125]. This so-called bacterial two-hybrid system has been used recently to characterize *E. coli* proteins involved in septum formation and cell division [130] and to detect premature stop codons in human genes (potentially useful for diagnosis of some hereditary cancers) by inserting the gene of interest between the two AC modules [131].

Use as a vaccine component

Adenylate cyclase toxin has not been included as a component of newer generation acellular pertussis vaccines, despite the observations that ACT is an important virulence factor for *B. pertussis*, purified ACT can protect mice against intranasal challenge with virulent *B. pertussis* [132] and anti-ACT antibodies enhance neutrophil phagocytosis of *B. pertussis*

through neutralization of ACT, preventing its inhibitory activity on these cells [133]. In addition, as described above, detoxified ACT is being developed as a possible vaccine vector molecule, so immune responses to the vector itself would be important. Two recent reports addressed the issue of the use of detoxified ACT as a pertussis vaccine component. In one, co-administration of detoxified ACT with acellular pertussis vaccine to mice significantly enhanced protection against *B. pertussis*, with adjuvant effects on both Th1 and Th2 immune responses [134]. In the other, however, detoxified ACT was found to block CR3 transiently and inhibit complement-dependent phagocytosis by human neutrophil-like cells [135], a potentially unwanted side effect. Whether this investigation will progress further to human clinical trials will probably depend upon a major push towards development of newer acellular pertussis vaccines.

Conclusion

Pertussis toxin and ACT are important virulence factors contributing to *B. pertussis* infection of the respiratory tract, at least in experimental models. Each is a complex toxin with multiple activities mediated either by an enzymatically active portion or a cell-binding domain that, in the case of ACT, can form pores in the cell membrane that affect cellular function and viability. Ongoing research is attempting to distinguish between the role of these different activities in various effects on cellular and immune functions. Details on the method of cell entry and membrane translocation by these toxins are still emerging, with apparently unique aspects of these mechanisms revealed in each case. Recent studies strongly indicate that their primary role in promoting *B. pertussis* infection is to suppress and modulate host immune responses. These effects can be seen at multiple levels, including inhibition of phagocyte recruitment and activity, inhibition and modulation of inflammatory responses stimulated through TLRs, modulation of cytokine and chemokine production and skewing of T-cell phenotype, inhibition and modulation of antigen-presenting cell activities and adaptive immune responses, effects on other immune-related diseases, and adjuvant properties for stimulation of responses to other antigens. Some of these properties may be shared and may overlap between the two toxins, and synergistic effects may also occur. PT is an important cell biology tool owing to its inhibition of Gi-coupled receptor signaling pathways, and its immunomodulatory properties have been utilized in autoimmunity research. ACT is also an important cell biology tool for several applications: its capacity to deliver a domain to the cytosol of mammalian cells has been utilized to internalize fused immunogenic epitopes and stimulate antigen presentation and immune responses, and has also been utilized as a reporter for protein translocation from bacteria to eukaryotic cells; and the modular structure of its AC domain has been utilized to analyze protein–protein interaction in a bacterial two-hybrid system.

Future perspective

Pertussis toxin and ACT are clearly important toxins for *B. pertussis* infection, and their role as virulence factors and in modulating host immune responses will continue to receive attention from the research community. In 5–10 years, we will have a more complete understanding of the range of effects on the immune response and how specific activities of the toxins contribute to these effects. For instance, specific inhibitory activities of PT on AM will be identified, and the role that various cytokines (whose production is induced by PT and ACT) play in protection against and clearance of the infection will be elucidated. The contribution of PTB activities to *B. pertussis* infection and immunomodulation of the host will also be studied.

However, an important question yet to be addressed is whether the activities of PT and ACT, particularly in regard to their effects on immune responses, also promote disease pathogenesis associated with pertussis. Human volunteer studies for pertussis are unlikely to be approved in the near future. The mouse model provides only very limited opportunity to study this aspect

of pertussis; thus, it is likely that in future years new animal models will be developed. One leading possibility is nonhuman primates, in which old data suggest that the human airway pathology of pertussis, including paroxysmal coughing, is reproduced by *B. pertussis* infection. Such a model will allow confirmation of the role of PT and ACT in *B. pertussis* infection and elucidation of specific effects of these toxins in pertussis disease pathogenesis. Ideally, this may also lead to identification of new approaches for therapeutic intervention in pertussis disease.

Executive summary

Cellular trafficking & activity of pertussis toxin

- Pertussis toxin (PT) utilizes a retrograde transport pathway through the Golgi complex and endoplasmic reticulum (ER) to gain entry to the cell cytosol.

- ATP binding to PT B oligomer (PTB) in the ER promotes holotoxin dissociation for further transport of the active subunit.

- PT active subunit is putatively transported from the ER to the cytosol by the endogenous ERAD pathway; however, convincing evidence supporting this has yet to be obtained.

Role of PT as a virulence factor: modulation of host immune responses

- PT plays an early role in infection by targeting resident airway macrophages.

- PT delays early neutrophil recruitment by inhibiting chemokine gene upregulation, but neutrophils play a protective role against *Bordetella pertussis* infection only in immune mice.

- PT stimulates production of Th1- and Th17-associated cytokines at the peak of infection, but the role of these cytokines in clearance and pathology is unclear.

- PT also inhibits adaptive immunity by effects on different cell types.

- Several lines of evidence suggest that PT is important in pertussis infection and disease in humans.

PTB binding activity

- Binding and signaling by PTB also elicits various cellular effects, though the role of these effects in *B. pertussis* infection is unclear.

- PTB inhibits HIV entry and replication in various cell types.

- PTB adjuvanticity for immune responses may be achieved by signaling through Toll-like receptor 4.

- PTB can also signal through the T-cell receptor to downregulate CXCR4 expression.

Role of PT in autoimmunity

- PT contributes to the pathogenesis of autoimmune diseases such as experimental autoimmune encephalitis and experimental autoimmune uveoretinitis through a variety of mechanisms.

- Whether *B. pertussis* infection can also enhance these diseases has not been addressed.

Structure, function & cellular activity of adenylate cyclase toxin

- Acylation of adenylate cyclase toxin (ACT) contributes to its activity by a variety of effects.

- Pore formation alone can be cytotoxic and is associated with ACT oligomers, while ACT monomers mediate adenylate cyclase domain translocation across the cell membrane.

- The structure of the repeat in toxin domain is altered by calcium binding after secretion from bacterial cells and contributes to pore formation.

- In addition to pore formation and adenylate cyclase activity increasing cAMP concentration, ATP depletion and unconventional calcium influx during translocation may also contribute to cytotoxic effects.

- ACT can also induce cytoskeletal changes and apoptosis.

Role of ACT as a virulence factor: modulation of host immune responses

- ACT targets neutrophils to promote *B. pertussis* infection.

- ACT modulates dendritic cell maturation and cytokine production, promoting IL-10 and -23 production.

- ACT induces many inflammatory genes in murine macrophages, including cyclooxygenase-2 expression.

Use of ACT as an antigen delivery tool

- Detoxified ACT can deliver MHC class I and II epitopes to antigen-presenting cells to stimulate protective T-cell responses *in vitro* and *in vivo*.

- This property of ACT was used to facilitate detection of latent TB infection from patient blood samples.

Use of ACT as a tool for intracellular protein detection & for protein–protein interaction

- Fusions with the AC domain of ACT are used to detect protein targeting to the mammalian cell cytosol, particularly by bacterial type III secretion systems.

- The modular structure of the AC domain is used in a bacterial two-hybrid genetic screen for protein–protein interaction.

Use of ACT as a vaccine component

- ACT has not been used as a component of acellular pertussis vaccines, although antibodies to ACT can enhance phagocytosis and detoxified ACT has adjuvant effects.

Other aspects of PT biology that will likely receive attention are:

- The pathway by which the S1 subunit gains access to the cytosol from the ER;

- The mechanism by which PT inhibits TLR4-mediated responses;

- Whether, and how, PTB activites, such as binding and signaling through TLR4 and the TCR, play a role in *B. pertussis* infection;

- Whether PT production during *B. pertussis* infection might exacerbate experimental autoimmune diseases.

Other aspects of ACT biology that will likely be addressed in the coming years are: first, the continued study of the structure–function relationship of the various domains of ACT; second,

the mechanism of cellular entry and the relationship between pore formation and membrane translocation; and third, the further development of ACT as a delivery vector for cellular immune responses for prophylactic, therapeutic and diagnostic applications, possibly extended to human clinical trials.

Acknowledgments

Bibliography

Papers of special note have been highlighted as:

- ▪ of interest
- ▪▪ of considerable interest

1. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev 2005;18:326–382. [PubMed: 15831828]

2. Wood N, McIntyre P. Pertussis: review of epidemiology, diagnosis, management and prevention. Paediatr Respir Rev 2008;9:201–211. [PubMed: 18694712]

3. Carbonetti NH. Immunomodulation in the pathogenesis of *Bordetella pertussis* infection and disease. Curr Opin Pharmacol 2007;7:272–278. [PubMed: 17418639]

4. Paddock CD, Sanden GN, Cherry JD, et al. Pathology and pathogenesis of fatal *Bordetella pertussis* infection in infants. Clin Infect Dis 2008;47:328–338. [PubMed: 18558873]

5. He Q, Mertsola J. Factors contributing to pertussis resurgence. Future Microbiol 2008;3:329–339. [PubMed: 18505398]

6. Mills KH. Immunity to *Bordetella pertussis*. Microbes Infect 2001;3:655–677. [PubMed: 11445452]

7. Verma A, Burns DL. Requirements for assembly of PtlH with the pertussis toxin transporter apparatus of *Bordetella pertussis*. Infect Immun 2007;75:2297–2306. [PubMed: 17339350]

8. Verma A, Cheung AM, Burns DL. Stabilization of the pertussis toxin secretion apparatus by the C terminus of PtlD. J Bacteriol 2008;190:7285–7290. [PubMed: 18723610]

9. Witvliet MH, Burns DL, Brennan MJ, Poolman JT, Manclark CR. Binding of pertussis toxin to eucaryotic cells and glycoproteins. Infect Immun 1989;57:3324–3330. [PubMed: 2478471]

10. Wong WS, Rosoff PM. Pharmacology of pertussis toxin B-oligomer. Can J Physiol Pharmacol 1996;74:559–564. [PubMed: 8884020]

11. Saukkonen K, Burnette WN, Mar VL, Masure HR, Tuomanen EI. Pertussis toxin has eukaryotic-like carbohydrate recognition domains. Proc Natl Acad Sci USA 1992;89:118–122. [PubMed: 1729677]

12. Stein PE, Boodhoo A, Armstrong GD, et al. Structure of a pertussis toxin–sugar complex as a model for receptor binding. Nat Struct Biol 1994;1:591–596. [PubMed: 7634099]

13. Katada T, Tamura M, Ui M. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch Biochem Biophys 1983;224:290–298. [PubMed: 6683482]

14. Moss J, Stanley SJ, Burns DL, et al. Activation by thiol of the latent NAD glycohydrolase and ADP-ribosyltransferase activities of *Bordetella pertussis* toxin (islet-activating protein). J Biol Chem 1983;258:11879–11882. [PubMed: 6311827]

15. Burns DL, Manclark CR. Adenine nucleotides promote dissociation of pertussis toxin subunits. J Biol Chem 1986;261:4324–4327. [PubMed: 3005329]

16. Hazes B, Boodhoo A, Cockle SA, Read RJ. Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. J Mol Biol 1996;258:661–671. [PubMed: 8637000]

17. Stein PE, Boodhoo A, Armstrong GD, Cockle SA, Klein MH, Read RJ. The crystal structure of pertussis toxin. Structure 1994;2:45–57. [PubMed: 8075982]

18. Pierce C, Klein N, Peters M. Is leukocytosis a predictor of mortality in severe pertussis infection? Intensive Care Med 2000;26:1512–1514. [PubMed: 11126265]

19. Mikelova LK, Halperin SA, Scheifele D, et al. Predictors of death in infants hospitalized with pertussis: a case–control study of 16 pertussis deaths in Canada. J Pediatr 2003;143:576–581. [PubMed: 14615725]

20. Trollfors B, Taranger J, Lagergard T, et al. A placebo-controlled trial of a pertussis-toxoid vaccine. N Engl J Med 1995;333:1045–1050. [PubMed: 7675047]

21. Plaut RD, Carbonetti NH. Retrograde transport of pertussis toxin in the mammalian cell. Cell Microbiol 2008;10:1130–1139. [PubMed: 18201245]

22. Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 2008;9:944–957. [PubMed: 19002207]

23. Worthington ZE, Carbonetti NH. Evading the proteasome: absence of lysine residues contributes to pertussis toxin activity by evasion of proteasome degradation. Infect Immun 2007;75:2946–2953. [PubMed: 17420233]

24. Pande AH, Moe D, Jamnadas M, Tatulian SA, Teter K. The pertussis toxin S1 subunit is a thermally unstable protein susceptible to degradation by the 20S proteasome. Biochemistry 2006;45:13734–13740. [PubMed: 17105192]

25. Munoz JJ, Arai H, Bergman RK, Sadowski PL. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect Immun 1981;33:820–826. [PubMed: 6269999]

26. Goodwin MS, Weiss AA. Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. Infect Immun 1990;58:3445–3447. [PubMed: 2401570]

27. Carbonetti NH, Artamonova GV, Andreasen C, Bushar N. Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of *Bordetella pertussis* infection of the respiratory tract. Infect Immun 2005;73:2698–2703. [PubMed: 15845471]

28. Carbonetti NH, Artamonova GV, Mays RM, Worthington ZE. Pertussis toxin plays an early role in respiratory tract colonization by *Bordetella pertussis*. Infect Immun 2003;71:6358–6366. [PubMed: 14573656] ▪▪ Established pertussis toxin (PT) as an important virulence factor for early respiratory tract infection by *Bordetella pertussis* that acts as a soluble factor on resident airway cells.

29. Carbonetti NH, Artamonova GV, Van Rooijen N, Ayala VI. Pertussis toxin targets airway macrophages to promote *Bordetella pertussis* infection of the respiratory tract. Infect Immun 2007;75:1713–1720. [PubMed: 17242062] ▪▪ Here resident airway macrophages were shown to be an important target for PT in promoting airway infection by *B. pertussis*.

30. Lattin J, Zidar DA, Schroder K, Kellie S, Hume DA, Sweet MJ. G-protein-coupled receptor expression, function, and signaling in macrophages. J Leukoc Biol 2007;82:16–32. [PubMed: 17456803]

31. Andreasen C, Carbonetti NH. Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to *Bordetella pertussis* respiratory tract infection in mice. Infect Immun 2008;76:5139–5148. [PubMed: 18765723] ▪ Reports the mechanism by which PT inhibits early neutrophil influx to the airways after *B. pertussis* infection.

32. Kirimanjeswara GS, Agosto LM, Kennett MJ, Bjornstad ON, Harvill ET. Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. J Clin Invest 2005;115:3594–3601. [PubMed: 16294220] ▪ PT is shown to inhibit antibody-mediated clearance of *B. pertussis* from the lower respiratory tract through a mechanism involving neutrophils, by inhibiting neutrophil recruitment to the airways.

33. Reutershan J, Basit A, Galkina EV, Ley K. Sequential recruitment of neutrophils into lung and bronchoalveolar lavage fluid in LPS-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol 2005;289:L807–L815. [PubMed: 15951336]

34. Pero RS, Borchers MT, Spicher K, et al. Gαi2-mediated signaling events in the endothelium are involved in controlling leukocyte extravasation. Proc Natl Acad Sci USA 2007;104:4371–4376. [PubMed: 17360531]

35. Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. J Immunol 2006;177:7980–7989. [PubMed: 17114471]

36. Ross PJ, Lavelle EC, Mills KH, Boyd AP. Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. Infect Immun 2004;72:1568–1579. [PubMed: 14977963]

37. Mann PB, Wolfe D, Latz E, Golenbock D, Preston A, Harvill ET. Comparative toll-like receptor 4-mediated innate host defense to *Bordetella* infection. Infect Immun 2005;73:8144–8152. [PubMed: 16299309]

38. Banus S, Stenger RM, Gremmer ER, et al. The role of Toll-like receptor-4 in pertussis vaccine-induced immunity. BMC Immunol 2008;9:21. [PubMed: 18498620]

39. Fedele G, Nasso M, Spensieri F, et al. Lipopolysaccharides from *Bordetella pertussis* and *Bordetella parapertussis* differently modulate human dendritic cell functions resulting in divergent prevalence of Th17-polarized responses. J Immunol 2008;181:208–216. [PubMed: 18566386]

40. Lentschat A, Karahashi H, Michelsen KS, et al. Mastoparan, a G protein agonist peptide, differentially modulates TLR4- and TLR2-mediated signaling in human endothelial cells and murine macrophages. J Immunol 2005;174:4252–4261. [PubMed: 15778388]

41. Fan H, Peck OM, Tempel GE, Halushka PV, Cook JA. Toll-like receptor 4 coupled GI protein signaling pathways regulate extracellular signal-regulated kinase phosphorylation and AP-1 activation independent of NFκB activation. Shock 2004;22:57–62. [PubMed: 15201703]

42. Andreasen C, Carbonetti NH. Role of neutrophils in response to *Bordetella pertussis* infection in mice. Infect Immun 2009;77:1182–1188. [PubMed: 19103765]

43. Andreasen C, Powell D, Carbonetti NH. Pertussis toxin stimulates IL-17 production in response to *Bordetella pertussis* infection in mice. PLoS ONE 2009;4:E7079. [PubMed: 19759900] ▪▪ This study demonstrated that PT promotes Th1 and Th17 inflammatory cytokine upregulation in the airways at the peak of *B. pertussis* infection.

44. Wolfe DN, Mann PB, Buboltz AM, Harvill ET. Delayed role of tumor necrosis factor-α in overcoming the effects of pertussis toxin. J Infect Dis 2007;196:1228–1236. [PubMed: 17955442]

45. Carbonetti NH, Artamonova GV, Andreasen C, Dudley E, Mays RM, Worthington ZE. Suppression of serum antibody responses by pertussis toxin after respiratory tract colonization by *Bordetella pertussis* and identification of an immunodominant lipoprotein. Infect Immun 2004;72:3350–3358. [PubMed: 15155640]

46. Shumilla JA, Lacaille V, Hornell TM, et al. *Bordetella pertussis* infection of primary human monocytes alters HLA-DR expression. Infect Immun 2004;72:1450–1462. [PubMed: 14977950]

47. Martino A, Volpe E, Auricchio G, Colizzi V, Baldini PM. Influence of pertussis toxin on CD1a isoform expression in human dendritic cells. J Clin Immunol 2006;26:153–159. [PubMed: 16598657]

48. Bouchez V, Brun D, Cantinelli T, Dore G, Njamkepo E, Guiso N. First report and detailed characterization of *B. pertussis* isolates not expressing pertussis toxin or pertactin. Vaccine 2009;27 (43):6034–6041. [PubMed: 19666155]

49. Mooi FR, van Loo IHM, van Gent M, et al. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 2009;15:1206–1213. [PubMed: 19751581]

50. Robbins JB, Schneerson R, Keith JM, Miller MA, Kubler-Kielb J, Trollfors B. Pertussis vaccine: a critique. Pediatr Infect Dis J 2009;28:237–241. [PubMed: 19165133]

51. Pizza M, Covacci A, Bartoloni A, et al. Mutants of pertussis toxin suitable for vaccine development. Science 1989;246:497–500. [PubMed: 2683073]

52. Alfano M, Schmidtmayerova H, Amella CA, Pushkarsky T, Bukrinsky M. The B-oligomer of pertussis toxin deactivates CC chemokine receptor 5 and blocks entry of M-tropic HIV-1 strains. J Exp Med 1999;190:597–605. [PubMed: 10477545]

53. Rizzi C, Alfano M, Bugatti A, Camozzi M, Poli G, Rusnati M. Inhibition of intra- and extra-cellular Tat function and HIV expression by pertussis toxin B-oligomer. Eur J Immunol 2004;34:530–536. [PubMed: 14768058]

54. Zocchi MR, Contini P, Alfano M, Poggi A. Pertussis toxin (PTX) B subunit and the nontoxic PTX mutant PT9K/129G inhibit Tat-induced TGF-β production by NK cells and TGF-β-mediated NK cell apoptosis. J Immunol 2005;174:6054–6061. [PubMed: 15879099]

55. Poggi A, Zocchi MR. HIV-1 Tat triggers TGF-β production and NK cell apoptosis that is prevented by pertussis toxin B. Clin Dev Immunol 2006;13:369–372. [PubMed: 17162379]

56. Jajoo S, Mukherjea D, Brewer GJ, Ramkumar V. Pertussis toxin B-oligomer suppresses human immunodeficiency virus-1 Tat-induced neuronal apoptosis through feedback inhibition of phospholipase C-β by protein kinase C. Neuroscience 2008;151:525–532. [PubMed: 18093742]

57. Rizzi C, Crippa MP, Jeeninga RE, et al. Pertussis toxin B-oligomer suppresses IL-6 induced HIV-1 and chemokine expression in chronically infected U1 cells via inhibition of activator protein 1. J Immunol 2006;176:999–1006. [PubMed: 16393986]

58. Hu Q, Younson J, Griffin GE, Kelly C, Shattock RJ. Pertussis toxin and its binding unit inhibit HIV-1 infection of human cervical tissue and macrophages involving a CD14 pathway. J Infect Dis 2006;194:1547–1556. [PubMed: 17083039]

59. Lapenta C, Spada M, Santini SM, et al. Pertussis toxin B-oligomer inhibits HIV infection and replication in hu-PBL-SCID mice. Int Immunol 2005;17:469–475. [PubMed: 15746245]

60. Alfano M, Grivel JC, Ghezzi S, et al. Pertussis toxin B-oligomer dissociates T cell activation and HIV replication in CD4 T cells released from infected lymphoid tissue. AIDS 2005;19:1007–1014. [PubMed: 15958831]

61. Ryan M, McCarthy L, Rappuoli R, Mahon BP, Mills KH. Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7–1, B7–2 and CD28. Int Immunol 1998;10:651–662. [PubMed: 9645613]

62. Wang ZY, Yang D, Chen Q, et al. Induction of dendritic cell maturation by pertussis toxin and its B subunit differentially initiate Toll-like receptor 4-dependent signal transduction pathways. Exp Hematol 2006;34:1115–1124. [PubMed: 16863919] ▪ Highlights the differences between PT and PT B oligomer in adjuvant effects through TLR4.

63. Nasso M, Fedele G, Spensieri F, et al. Genetically detoxified pertussis toxin induces Th1/Th17 immune response through MAPKs and IL-10-dependent mechanisms. J Immunol 2009;183:1892–1899. [PubMed: 19596995]

64. Schneider OD, Weiss AA, Miller WE. Pertussis toxin utilizes proximal components of the T-cell receptor complex to initiate signal transduction events in T cells. Infect Immun 2007;75:4040–4049. [PubMed: 17562776] ▪ T-cell receptor was identified as a surface complex mediating PT B oligomer signaling in cells.

65. Schneider OD, Weiss AA, Miller WE. Pertussis toxin signals through the TCR to initiate cross-desensitization of the chemokine receptor CXCR4. J Immunol 2009;182:5730–5739. [PubMed: 19380820]

66. Munoz JJ, Bernard CC, Mackay IR. Elicitation of experimental allergic encephalomyelitis (EAE) in mice with the aid of pertussigen. Cell Immunol 1984;83:92–100. [PubMed: 6607126]

67. Kugler S, Bocker K, Heusipp G, Greune L, Kim KS, Schmidt MA. Pertussis toxin transiently affects barrier integrity, organelle organization and transmigration of monocytes in a human brain microvascular endothelial cell barrier model. Cell Microbiol 2007;9:619–632. [PubMed: 17002784]

68. Kerfoot SM, Long EM, Hickey MJ, et al. TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. J Immunol 2004;173:7070–7077. [PubMed: 15557205]

69. Chen X, Winkler-Pickett RT, Carbonetti NH, et al. Pertussis toxin as an adjuvant suppresses the number and function of CD4^{+}CD25^{+} T regulatory cells. Eur J Immunol 2006;36:671–680. [PubMed: 16479542]

70. Cassan C, Piaggio E, Zappulla JP, et al. Pertussis toxin reduces the number of splenic Foxp3^{+} regulatory T cells. J Immunol 2006;177:1552–1560. [PubMed: 16849462]

71. Amend B, Doster H, Lange C, et al. Induction of autoimmunity by expansion of autoreactive CD4^{+}CD62[Low] cells *in vivo*. J Immunol 2006;177:4384–4390. [PubMed: 16982873]

72. Chen X, Howard OM, Oppenheim JJ. Pertussis toxin by inducing IL-6 promotes the generation of IL-17-producing CD4 cells. J Immunol 2007;178:6123–6129. [PubMed: 17475838]

73. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 2006;177:566–573. [PubMed: 16785554]

74. Millward JM, Caruso M, Campbell IL, Gauldie J, Owens T. IFN-γ-induced chemokines synergize with pertussis toxin to promote T cell entry to the central nervous system. J Immunol 2007;178:8175–8182. [PubMed: 17548656]

75. Fujimoto C, Shi G, Gery I. Microbial products trigger autoimmune ocular inflammation. Ophthalmic Res 2008;40:193–199. [PubMed: 18421238]

76. Fujimoto C, Yu CR, Shi G, et al. Pertussis toxin is superior to TLR ligands in enhancing pathogenic autoimmunity, targeted at a neo-self antigen, by triggering robust expansion of Th1 cells and their cytokine production. J Immunol 2006;177:6896–6903. [PubMed: 17082604] ▪ Highlights the potency of PT as an adjuvant for autoimmune effects through induction of cytokine production.

77. Vojtova J, Kamanova J, Sebo P. *Bordetella* adenylate cyclase toxin: a swift saboteur of host defense. Curr Opin Microbiol 2006;9:69–75. [PubMed: 16406775]

78. Gray MC, Donato GM, Jones FR, Kim T, Hewlett EL. Newly secreted adenylate cyclase toxin is responsible for intoxication of target cells by *Bordetella pertussis*. Mol Microbiol 2004;53:1709–1719. [PubMed: 15341649]

79. Guermonprez P, Khelef N, Blouin E, et al. The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the α(M) β(2) integrin (CD11b/CD18). J Exp Med 2001;193:1035–1044. [PubMed: 11342588]

80. Morova J, Osicka R, Masin J, Sebo P. RTX cytotoxins recognize β2 integrin receptors through *N*-linked oligosaccharides. Proc Natl Acad Sci USA 2008;105:5355–5360. [PubMed: 18375764]

81. Guo Q, Shen Y, Lee YS, Gibbs CS, Mrksich M, Tang WJ. Structural basis for the interaction of *Bordetella pertussis* adenylyl cyclase toxin with calmodulin. EMBO J 2005;24:3190–3201. [PubMed: 16138079]

82. Guo Q, Jureller JE, Warren JT, Solomaha E, Florian J, Tang WJ. Protein–protein docking and analysis reveal that two homologous bacterial adenylyl cyclase toxins interact with calmodulin differently. J Biol Chem 2008;283:23836–23845. [PubMed: 18583346]

83. El-Azami-El-Idrissi M, Bauche C, Loucka J, et al. Interaction of *Bordetella pertussis* adenylate cyclase with CD11b/CD18: role of toxin acylation and identification of the main integrin interaction domain. J Biol Chem 2003;278:38514–38521. [PubMed: 12885782]

84. Masin J, Basler M, Knapp O, et al. Acylation of lysine 860 allows tight binding and cytotoxicity of *Bordetella* adenylate cyclase on CD11b-expressing cells. Biochemistry 2005;44:12759–12766. [PubMed: 16171390]

85. Lee SJ, Gray MC, Zu K, Hewlett EL. Oligomeric behavior of *Bordetella pertussis* adenylate cyclase toxin in solution. Arch Biochem Biophys 2005;438:80–87. [PubMed: 15878155]

86. Vojtova-Vodolanova J, Basler M, Osicka R, et al. Oligomerization is involved in pore formation by *Bordetella* adenylate cyclase toxin. Faseb J 2009;23:2831–2843. [PubMed: 19417082] ▪▪ Indicates that adenylate cyclase toxin (ACT) oligomers are associated with pore formation while ACT monomers mediate adenylate cyclase domain translocation.

87. Basler M, Knapp O, Masin J, et al. Segments crucial for membrane translocation and pore-forming activity of Bordetella adenylate cyclase toxin. J Biol Chem 2007;282:12419–12429. [PubMed: 17347146]

88. Powthongchin B, Angsuthanasombat C. Effects on haemolytic activity of single proline substitutions in the *Bordetella pertussis* CyaA pore-forming fragment. Arch Microbiol 2009;191:1–9. [PubMed: 18712361]

89. Knapp O, Maier E, Masin J, Sebo P, Benz R. Pore formation by the *Bordetella* adenylate cyclase toxin in lipid bilayer membranes: role of voltage and pH. Biochim Biophys Acta 2008;1778:260–269. [PubMed: 17976530]

90. Lilie H, Haehnel W, Rudolph R, Baumann U. Folding of a synthetic parallel β-roll protein. FEBS Lett 2000;470:173–177. [PubMed: 10734229]

91. Bauche C, Chenal A, Knapp O, et al. Structural and functional characterization of an essential RTX subdomain of *Bordetella pertussis* adenylate cyclase toxin. J Biol Chem 2006;281:16914–16926. [PubMed: 16627468]

92. Chenal A, Guijarro JI, Raynal B, Delepierre M, Ladant D. RTX calcium binding motifs are intrinsically disordered in the absence of calcium: implication for protein secretion. J Biol Chem 2009;284:1781–1789. [PubMed: 19015266]

93. Hewlett EL, Donato GM, Gray MC. Macrophage cytotoxicity produced by adenylate cyclase toxin from *Bordetella pertussis*: more than just making cyclic AMP! Mol Microbiol 2006;59:447–459. [PubMed: 16390441]

94. Basler M, Masin J, Osicka R, Sebo P. Pore-forming and enzymatic activities of *Bordetella pertussis* adenylate cyclase toxin synergize in promoting lysis of monocytes. Infect Immun 2006;74:2207–2214. [PubMed: 16552051]

95. Fiser R, Masin J, Basler M, et al. Third activity of *Bordetella adenylate* cyclase (AC) toxin-hemolysin. Membrane translocation of AC domain polypeptide promotes calcium influx into CD11b$^+$ monocytes independently of the catalytic and hemolytic activities. J Biol Chem 2007;282:2808–2820. [PubMed: 17148436] ▪ A novel cellular activity of ACT was identified in this report.

96. Vojtova J, Kofronova O, Sebo P, Benada O. *Bordetella* adenylate cyclase toxin induces a cascade of morphological changes of sheep erythrocytes and localizes into clusters in erythrocyte membranes. Microsc Res Tech 2006;69:119–129. [PubMed: 16456835]

97. Ohnishi H, Miyake M, Kamitani S, Horiguchi Y. The morphological changes in cultured cells caused by *Bordetella pertussis* adenylate cyclase toxin. FEMS Microbiol Lett 2008;279:174–179. [PubMed: 18179583]

98. Kamanova J, Kofronova O, Masin J, et al. Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. J Immunol 2008;181:5587–5597. [PubMed: 18832717]

99. Johansson D, Bergstrom P, Henriksson R, et al. Adenylate cyclase toxin from *Bordetella pertussis* enhances cisplatin-induced apoptosis to lung cancer cells *in vitro*. Oncol Res 2006;15:423–430. [PubMed: 16555548]

100. Cheung GY, Kelly SM, Jess TJ, et al. Functional and structural studies on different forms of the adenylate cyclase toxin of *Bordetella pertussis*. Microb Pathog 2009;46:36–42. [PubMed: 18992319]

101. Confer D, Eaton J. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science 1982;217:948–950. [PubMed: 6287574]

102. Gross MK, Au DC, Smith AL, Storm DR. Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional cyaA toxin of *Bordetella pertussis* abolish virulence. Proc Natl Acad Sci USA 1992;89:4898–4902. [PubMed: 1594590]

103. Khelef N, Sakamoto H, Guiso N. Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb Pathog 1992;12:227–235. [PubMed: 1614333]

104. Gueirard P, Druilhe A, Pretolani M, Guiso N. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo*. Infect Immun 1998;66:1718–1725. [PubMed: 9529102]

105. Khelef N, Guiso N. Induction of macrophage apoptosis by *Bordetella pertussis* adenylate cyclase-hemolysin. FEMS Microbiol Lett 1995;134:27–32. [PubMed: 8593951]

106. Khelef N, Zychlinsky A, Guiso N. *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun 1993;61:4064–4071. [PubMed: 8406793]

107. Mobberley-Schuman PS, Weiss AA. Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. Infect Immun 2005;73:7317–7323. [PubMed: 16239529]

108. Veal-Carr WL, Stibitz S. Demonstration of differential virulence gene promoter activation *in vivo* in *Bordetella pertussis* using RIVET. Mol Microbiol 2005;55:788–798. [PubMed: 15661004]

109. Boyd AP, Ross PJ, Conroy H, Mahon N, Lavelle EC, Mills KH. *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. J Immunol 2005;175:730–738. [PubMed: 16002668]

110. Skinner JA, Reissinger A, Shen H, Yuk MH. *Bordetella* type III secretion and adenylate cyclase toxin synergize to drive dendritic cells into a semimature state. J Immunol 2004;173:1934–1940. [PubMed: 15265927]

111. Spensieri F, Fedele G, Fazio C, et al. *Bordetella pertussis* inhibition of interleukin-12 (IL-12) p70 in human monocyte-derived dendritic cells blocks IL-12 p35 through adenylate cyclase toxin-dependent cyclic AMP induction. Infect Immun 2006;74:2831–2838. [PubMed: 16622221]

112. Hickey FB, Brereton CF, Mills KH. Adenylate cyclase toxin of *Bordetella pertussis* inhibits TLR-induced IRF-1 and IRF-8 activation and IL-12 production and enhances IL-10 through MAPK activation in dendritic cells. J Leukoc Biol 2008;84:234–243. [PubMed: 18401006] ▪▪ Identifies mechanisms by which ACT mediates immunomodulation through activity on dendritic cells.

113. Paccani SR, Dal Molin F, Benagiano M, et al. Suppression of T-lymphocyte activation and chemotaxis by the adenylate cyclase toxin of *Bordetella pertussis*. Infect Immun 2008;76:2822–2832. [PubMed: 18426886]

114. Rossi Paccani S, Benagiano M, Capitani N, et al. The adenylate cyclase toxins of *Bacillus anthracis* and *Bordetella pertussis* promote Th2 cell development by shaping T cell antigen receptor signaling. PLoS Pathog 2009;5:e1000325. [PubMed: 19266022]

115. Perkins DJ, Gray MC, Hewlett EL, Vogel SN. *Bordetella pertussis* adenylate cyclase toxin (ACT) induces cyclooxygenase-2 (COX-2) in murine macrophages and is facilitated by ACT interaction with CD11b/CD18 (Mac-1). Mol Microbiol 2007;66:1003–1015. [PubMed: 17927697]

116. Cheung GY, Dickinson P, Sing G, et al. Transcriptional responses of murine macrophages to the adenylate cyclase toxin of *Bordetella pertussis*. Microb Pathog 2008;44:61–70. [PubMed: 17890046] ▪▪ Describes a multitude of effects of ACT on transcriptional responses in macrophages, including upregulation of many inflammatory genes and downregulation of numerous cell-proliferation genes.

117. Schlecht G, Loucka J, Najar H, Sebo P, Leclerc C. Antigen targeting to CD11b allows efficient presentation of CD4+ and CD8+ T cell epitopes and *in vivo* Th1-polarized T cell priming. J Immunol 2004;173:6089–6097. [PubMed: 15528345]

118. Simsova M, Sebo P, Leclerc C. The adenylate cyclase toxin from *Bordetella pertussis* – a novel promising vehicle for antigen delivery to dendritic cells. Int J Med Microbiol 2004;293:571–576. [PubMed: 15149033]

119. Fayolle C, Bauche C, Ladant D, Leclerc C. *Bordetella pertussis* adenylate cyclase delivers chemically coupled CD8+ T-cell epitopes to dendritic cells and elicits CTL *in vivo*. Vaccine 2004;23:604–614. [PubMed: 15542180]

120. Mascarell L, Bauche C, Fayolle C, et al. Delivery of the HIV-1 Tat protein to dendritic cells by the CyaA vector induces specific Th1 responses and high affinity neutralizing antibodies in non human primates. Vaccine 2006;24:3490–3499. [PubMed: 16524647]

121. Mascarell L, Fayolle C, Bauche C, Ladant D, Leclerc C. Induction of neutralizing antibodies and Th1-polarized and CD4-independent CD8+ T-cell responses following delivery of human immunodeficiency virus type 1 Tat protein by recombinant adenylate cyclase of *Bordetella pertussis*. J Virol 2005;79:9872–9884. [PubMed: 16014948]

122. Tartz S, Russmann H, Kamanova J, et al. Complete protection against *P. berghei* malaria upon heterologous prime/boost immunization against circumsporozoite protein employing *Salmonella* type III secretion system and *Bordetella* adenylate cyclase toxoid. Vaccine 2008;26:5935–5943. [PubMed: 18804138]

123. Connell TG, Shey MS, Seldon R, et al. Enhanced *ex vivo* stimulation of *Mycobacterium tuberculosis*-specific T cells in human immunodeficiency virus-infected persons via antigen delivery by the *Bordetella pertussis* adenylate cyclase vector. Clin Vaccine Immunol 2007;14:847–854. [PubMed: 17522328]

124. Wilkinson KA, Simsova M, Scholvinck E, et al. Efficient *ex vivo* stimulation of *Mycobacterium tuberculosis*-specific T cells by genetically detoxified *Bordetella pertussis* adenylate cyclase antigen toxoids. Infect Immun 2005;73:2991–2998. [PubMed: 15845506]

125. Dautin N, Karimova G, Ladant D. *Bordetella pertussis* adenylate cyclase toxin: a versatile screening tool. Toxicon 2002;40:1383–1387. [PubMed: 12368108]

126. Geddes K, Worley M, Niemann G, Heffron F. Identification of new secreted effectors in *Salmonella enterica* serovar Typhimurium. Infect Immun 2005;73:6260–6271. [PubMed: 16177297]

127. Gendlina I, Held KG, Bartra SS, et al. Identification and type III-dependent secretion of the Yersinia pestis insecticidal-like proteins. Mol Microbiol 2007;64:1214–1227. [PubMed: 17542916]

128. Furutani A, Takaoka M, Sanada H, et al. Identification of novel type III secretion effectors in *Xanthomonas oryzae pv. oryzae*. Mol Plant Microbe Interact 2009;22:96–106. [PubMed: 19061406]

129. Miyake M, Sakane S, Kobayashi C, et al. A colorimetric assay for studying effector secretion through the bacterial type III secretion system. FEMS Microbiol Lett 2008;278:36–42. [PubMed: 17995954]

130. Karimova G, Robichon C, Ladant D. Characterization of YmgF, a 72-residue inner membrane protein that associates with the *Escherichia coli* cell division machinery. J Bacteriol 2009;191:333–346. [PubMed: 18978050]

131. Real SM, Marzese DM, Gomez LC, Mayorga LS, Roque M. Development of a premature stop codon-detection method based on a bacterial two-hybrid system. BMC Biotechnol 2006;6:38. [PubMed: 16948859]

132. Guiso N, Szatanik M, Rocancourt M. Protective activity of *Bordetella* adenylate cyclase-hemolysin against bacterial colonization. Microb Pathog 1991;11:423–431. [PubMed: 1795632]

133. Weingart CL, Mobberley-Schuman PS, Hewlett EL, Gray MC, Weiss AA. Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. Infect Immun 2000;68:7152–7155. [PubMed: 11083845]

134. Cheung GY, Xing D, Prior S, Corbel MJ, Parton R, Coote JG. Effect of different forms of adenylate cyclase toxin of *Bordetella pertussis* on protection afforded by an acellular pertussis vaccine in a murine model. Infect Immun 2006;74:6797–6805. [PubMed: 16982827]

135. Prior S, Fleck RA, Gillett ML, et al. Evaluation of adenyl cyclase toxin constructs from *Bordetella pertussis* as candidate vaccine components in an *in vitro* model of complement-dependent intraphagocytic killing. Vaccine 2006;24:4794–4803. [PubMed: 16675078]