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ROLE OF MAJOR TOXIN VIRULENCE FACTORS IN PERTUSSIS INFECTION AND DISEASE PATHOGENESIS

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Abstract

Bordetella pertussis produces several toxins that affect host-pathogen interactions. Of these, the major toxins that contribute to pertussis infection and disease are pertussis toxin, adenylate cyclase toxin-hemolysin and tracheal cytotoxin. Pertussis toxin is a multi-subunit protein toxin that inhibits host G protein-coupled receptor signaling, causing a wide array of effects on the host. Adenylate cyclase toxin-hemolysin is a single polypeptide, containing an adenylate cyclase enzymatic domain coupled to a hemolysin domain, that primarily targets phagocytic cells to inhibit their antibacterial activities. Tracheal cytotoxin is a fragment of peptidoglycan released by *B. pertussis* that elicits damaging inflammatory responses in host cells. This chapter describes these three virulence factors of *B. pertussis*, summarizing background information and focusing on the role of each toxin in infection and disease pathogenesis, as well as their role in pertussis vaccination.

Keywords

Bordetella toxins; Pertussis toxin; Adenylate cyclase toxin-hemolysin; Tracheal Cytotoxin; Pertussis pathogenesis

1. PERTUSSIS TOXIN

1.1 Background

Pertussis toxin (Agarwal et al.) is a multi-subunit (AB₅) protein toxin secreted by *B. pertussis*. PT binds mammalian cell surface glycosylated molecules (Witvliet et al. 1989) in a non-saturable and non-specific manner (Finck-Barbancon and Barbieri 1996), indicating lack of a specific receptor. PT is endocytosed and transported by the retrograde pathway to the endoplasmic reticulum (el Baya et al. 1997; Plaut and Carbonetti 2008; Plaut et al. 2016), from where the A subunit (S1) translocates to the cytosol (Hazes et al. 1996; Pande et al. 2006; Worthington and Carbonetti 2007). In the cytosol, S1 ADP-ribosylates the alpha subunit of heterotrimeric G proteins of the G_{i/o} class in mammalian cells, inhibiting activation of these G proteins by ligand-bound G protein-coupled receptors (GPCR) (Katada 2012). This modification has multiple effects on host cell activities, since many different GPCRs couple to G_i proteins. Binding of the PT B pentamer to mammalian cells elicits

various signaling effects independently of the enzymatic activity of S1, but these effects are relatively transient and concentration-dependent (Carbonetti 2010; Mangmool and Kurose 2011; Wong and Rosoff 1996; Schneider et al. 2009; Zocchi et al. 2005) and it is unclear whether this binding and signaling activity of PT is relevant in vivo. PT is an important virulence factor for *B. pertussis* and is central to the pathogenesis of pertussis infection and disease (as described below) and a detoxified form of PT is an important component of currently used acellular pertussis vaccines (aPV) (Coutte and Loch 2015).

1.2 Role in infection and disease pathogenesis

1.2.1 Systemic and local effects of PT—Systemic administration of purified PT to experimental animals has a variety of biological and toxic effects (Munoz et al. 1981). The most important of these is leukocytosis (Morse and Morse 1976; Munoz et al. 1981; Hinds et al. 1996; Nogimori et al. 1984), a large increase in the number of circulating white blood cells that is also a prominent feature in human infants with pertussis and high levels of which correlate with fatal outcome (Winter et al. 2015). PT likely induces leukocytosis through a number of mechanisms (Carbonetti 2016), including reduced expression of leukocyte surface adhesion molecules such as LFA-1 (Schenkel and Pauza 1999) and CD62L (Hodge et al. 2003; Hudnall and Molina 2000), inhibition of LFA-1-dependent lymphocyte arrest on lymph node high endothelial venules (Bargatze and Butcher 1993; Warnock et al. 1998), and inhibition of chemokine receptor signaling affecting leukocyte migration (Beck et al. 2014; Pham et al. 2008). PT treatment of experimental animals also has adverse effects on control of heart rate and other cardiac functions, independently of leukocytosis (Grimm et al. 1998; Wainford et al. 2008; Adamson et al. 1993; Zheng et al. 2005). Another effect of PT is reduction of vascular barrier integrity (Dudek et al. 2007), which may contribute to the pathogenesis of experimental autoimmune encephalitis (EAE) in animal models (Bennett et al. 2010), several of which require PT as an adjuvant to stimulate disease (Munoz et al. 1984; Arimoto et al. 2000; Zhao et al. 2008). This led to the recent speculation that PT effects from sub-clinical pertussis infections may be a contributor to exacerbations of multiple sclerosis in humans (Rubin and Glazer 2016). Other effects of systemic administration of PT include vasoactive sensitization to histamine (Munoz et al. 1981) and induction of insulinemia/hypoglycemia (Yajima et al. 1978), although whether these are relevant to pertussis disease in humans is not clear.

The extent to which PT contributes to the respiratory pathology of pertussis is less clear. Experimental animals, such as mice and guinea pigs, do not cough when administered purified PT (Hewitt and Canning 2010) but baboons experimentally-infected with *B. pertussis* suffer the typical severe paroxysmal pertussis cough (Warfel et al. 2012). However, baboons infected with PT-deficient strains of *B. pertussis* show no cough symptoms at all, despite being colonized for the same duration as wild type *B. pertussis*-infected animals (Merkel, unpublished data). This may be because PT is necessary to induce robust respiratory inflammation, as demonstrated in mouse models (Connelly et al. 2012; Khelef et al. 1994), which may be an important contributor to the cough pathology (see more below). In addition, data from natural and volunteer *B. pertussis* infections indicate that PT plays a role in respiratory pathology. For instance, a PT-deficient strain isolated from a 3-month-old unvaccinated infant in France was associated with a relatively mild and short time course of

disease (Bouchez et al. 2009). Also, human volunteers intranasally inoculated with the candidate pertussis vaccine strain BPZE1, which expresses a genetically inactivated form of PT (Mielcarek et al. 2006), did not cough (Thorstensson et al. 2014), although this strain contains two other modifications in potential virulence factors. The paroxysmal nature of pertussis cough may also be an effect of PT activity, since PT can inhibit desensitization of receptors stimulated by tussive agents (Maher et al. 2011), thereby preventing the cessation of the coughing response. In addition, PT effects in experimental animals are long-lived (Carbonetti et al. 2003; Carbonetti et al. 2007) and so the longevity of pertussis cough may also be an effect of PT activity.

Another important activity of PT is likely in promoting fatal pertussis infection in young infants. PT induces leukocytosis and there is a significant correlation between high levels of leukocytosis and fatal outcome in these infants (Rowlands et al. 2010; Surridge et al. 2007; Pierce et al. 2000; Winter et al. 2015). Animal models provide additional evidence supporting the role of PT in promoting fatal outcome in pertussis. For example, PT is required for lethality of *B. pertussis* infection in neonatal Balb/c (Weiss and Goodwin 1989) and C57BL/6 mice (Scanlon et al. 2017). However, the specific mechanisms by which PT promotes lethality in pertussis remain to be determined and probably involve pathologies beyond leukocytosis and respiratory effects.

1.2.2 Effects of PT on *B. pertussis* colonization and host immune responses

—Most of our understanding of the effects of PT on colonization and immune responses is derived from mouse model experiments, with some recent data from baboon studies, by comparing infection with isogenic strains differing only in PT production. In mice, PT clearly promotes colonization of the respiratory tract, presumably through effects on immune responses (Carbonetti et al. 2003; Connelly et al. 2012). PT inhibits early innate immune defenses, including recruitment of neutrophils to *B. pertussis*-infected lungs (Carbonetti et al. 2005; Carbonetti et al. 2003; Kirimanjeswara et al. 2005; Andreassen and Carbonetti 2008) and anti-bacterial activity of resident airway macrophages (Carbonetti et al. 2007). PT also inhibits some aspects of adaptive immunity, including migration of dendritic cells in response to lymphatic chemokines (Fedele et al. 2011) and generation of serum antibody responses to *B. pertussis* during infection (Carbonetti et al. 2004; Mielcarek et al. 1998). However, at the peak of infection PT promotes lung inflammation (Connelly et al. 2012; Khelef et al. 1994; Andreassen et al. 2009), probably through a variety of mechanisms. One such mechanism may involve the PT-dependent increased expression of the epithelial anion exchanger pendrin, which promotes lung inflammatory pathology during *B. pertussis* infection (Scanlon et al. 2014). Another mechanism involves PT-dependent inhibition of the resolution of lung inflammation (Connelly et al. 2012), possibly by inhibiting activity of specialized pro-resolving lipid mediators such as resolvins and lipoxins (Levy and Serhan 2014), that signal through PT-sensitive GPCRs (Maddox et al. 1997; Krishnamoorthy et al. 2010; Chattopadhyay et al. 2018; Jo et al. 2016).

It is unclear whether lung inflammatory pathology correlates with the severe cough of human pertussis, but the recently developed baboon model of pertussis may shed some light on this. Baboons cough paroxysmally when infected with a clinical isolate of *B. pertussis* (Warfel et al. 2012; Warfel and Merkel 2014) although, as stated above, they do not develop

cough when infected with an isogenic PT-deficient strain. In addition, PT production was associated with cough in rats experimentally infected with *B. pertussis* (Parton et al. 1994). Even though PT may not be the direct cause of pertussis cough, these findings indicate that there is a strong association between PT production and cough responses, which may be due to the exacerbated inflammatory responses at the peak of infection that are promoted by PT.

1.3 PT as a pertussis vaccine component and therapeutic target

Since the move to aPV in most countries in recent years, a detoxified form of PT has been a component of all of these vaccines, in recognition of its role as a protective antigen against severe disease in infants (Coutte and Locht 2015). Although most of these vaccines contain other pertussis antigens, a monocomponent detoxified PT vaccine is used in Denmark (Thierry-Carstensen et al. 2013). Since pertussis has remained under control in Denmark (Dalby et al. 2016), this indicates that immune responses to this single component can protect a population from pertussis disease effectively. In addition, in the baboon model a monocomponent PT vaccine was sufficient to protect infant animals born to vaccinated mothers from pertussis disease (Kapil et al. 2018).

PT may also be an important target for therapy against pertussis. In one trial, administration of intravenous pertussis immunoglobulin (P-IGIV), which contains high levels of anti-PT antibodies, resulted in significant reduction in leukocytosis in infants with pertussis, and reduced paroxysmal coughing and bradycardic episodes (Bruss et al. 1999). In animal models, treatment with humanized murine monoclonal antibodies specific for PT was more effective than P-IGIV in preventing leukocytosis in mice and reduced leukocytosis when administered therapeutically to infected baboons (Nguyen et al. 2015), highlighting the therapeutic potential of this approach. Very recently, inhibitors of cyclophilins, such as cyclosporine A, were found to inhibit PT activity in cultured cells (Ernst et al. 2018), representing an additional therapeutic strategy targeting PT.

2. ADENYLATE CYCLASE TOXIN-HEMOLYSIN

2.1 Background

Adenylate cyclase toxin-hemolysin (CyaA, ACT or AC-Hly) is a repeat-in-toxin (RTX) cytotoxin expressed by three closely related *Bordetella* species, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (Endoh et al. 1980; Hewlett et al. 1976; Glaser et al. 1988a). This 1706 amino acid polypeptide is encoded by *cyaA* in an operon containing the type I secretion apparatus genes *cyaB*, *cyaD* and *cyaE* (Glaser et al. 1988b). CyaA contains an N-terminal adenylate cyclase (AC) enzyme domain of 364 amino acid residues and a C-terminal RTX hemolysin moiety of ~1300 residues (Glaser et al. 1988b). The hemolysin moiety is comprised of a hydrophobic pore-forming domain (Benz et al. 1994), an active domain containing two posttranslationally acylated lysine residues (Lys 860 and Lys 983) (Hackett et al. 1994; Hackett et al. 1995), a receptor binding RTX domain with characteristic calcium-binding glycine- and aspartate-rich nonapeptide repeats (Rose et al. 1995) and a C-terminal secretion signal for the type I secretion system (Bumba et al. 2016; Sebo and Ladant 1993). This toxin plays a key role in establishing *B. pertussis* infection. Activities mediated by both the AC and hemolysin domains of CyaA function to subvert host innate

immunity and thereby facilitate initial colonization (as described below). Current formulations of acellular pertussis vaccines do not contain CyaA. However, recently a focus on research elucidating the structure-function relationship of CyaA has emerged, with the aim of using this new understanding to rationally develop CyaA vaccine candidates (Cheung et al. 2006; Osickova et al. 2010; Boehm et al. 2018).

2.2 Role in infection and disease pathogenesis

2.2.1 Host cell subversion by CyaA—First described in 1976 as both a soluble and bacterial cell-associated enzyme (Hewlett et al. 1976), CyaA physically associates with filamentous haemagglutinin (FHA) to mediate toxin retention on the bacterial surface (Zaretzky et al. 2002). Interestingly, target cell intoxication is not dependent on surface-associated CyaA and instead newly secreted CyaA is necessary (Gray et al. 2004). However, unlike PT, which acts conventionally as a soluble factor (described above), exogenous administration of recombinant CyaA fails to rescue a “wild type” *B. pertussis* phenotype in mice infected with a *cyaA*-deficient strain (Carbonetti et al. 2005). In vitro studies have demonstrated rapid aggregation of CyaA in solution (Rogel et al. 1991). Hence, CyaA is hypothesized to be a short-lived toxin acting in close proximity to the bacterium (Vojtova et al. 2006) but the exact role of soluble vs bacterial-associated CyaA remains to be determined. Given that CyaA functions at the site of the bacterial-host interface, this toxin does not contribute to systemic pathologies during *B. pertussis* infection.

At the site of infection, locally secreted CyaA performs a number of functions to subvert host cell biology and potentiate cell death. Upon insertion into the target cell membrane, CyaA takes on one of two conformations (Osickova et al. 1999). It is proposed that a monomeric form of CyaA acts to translocate the catalytic AC enzyme domain into the host cell cytosol, whilst oligomerization of CyaA potentiates the formation of a hemolytic pore and that actions performed by the CyaA monomer improve the efficacy of the hemolysin moiety (Osickova et al. 1999; Fiser et al. 2012). The exact mechanism by which CyaA translocates its AC domain into the host cytosol is still under investigation. Recently, recombinant CyaA has been shown to display calcium-dependent phospholipase A (PLA) activity and this mechanism has been associated with CyaA-induced cytotoxicity in macrophages and macrophage release of free fatty acids (Gonzalez-Bullon et al. 2017). Ostolaza et al. suggest that CyaA-PLA acts to remodel the host cell membrane (releasing membrane fatty acids and lysophospholipids), generating a “toroidal pore” that facilitates transport of the AC domain (Ostolaza et al. 2017). However, whether CyaA itself, and not an *E. coli*-derived contaminant, possesses PLA activity has been contested (Masin et al. 2018; Ostolaza 2018), hence more studies are required to elucidate the contribution of PLA activity to CyaA biology. Translocation of the AC domain is concomitant with Ca^{2+} influx in the host cell (Fiser et al. 2007). This process is required to activate calpain-mediated processing of CyaA and liberation of the AC domain into the cytosol (Bumba et al. 2010; Uribe et al. 2013). In addition, CyaA-driven Ca^{2+} influx alters endocytic trafficking in the host cell membrane and limits macropinocytic removal of pore-forming oligomerized CyaA (Fiser et al. 2012). In the cytosol, AC binds host calmodulin and catalyzes the rapid, unregulated conversion of cytoplasmic ATP into cAMP (Wolff et al. 1980; Hanski and Farfel 1985; Guo et al. 2005). CyaA-induced accumulation of cAMP prevents bactericidal

activities of phagocytes by inhibiting oxidative burst and phagocytosis (Confer and Eaton 1982; Pearson et al. 1987; Kamanova et al. 2008; Friedman et al. 1987; Cerny et al. 2015), and modulates innate immune cell activation by inhibiting phagocyte maturation and suppressing the expression of proinflammatory cytokines and chemokines (Boyd et al. 2005; Njamkepo et al. 2000; Ross et al. 2004; Fedele et al. 2010; Spensieri et al. 2006). In addition, elevated cAMP levels inhibit the formation of neutrophil extracellular traps (NETs) and neutrophil apoptosis (Eby et al. 2014), whilst also promoting *B. pertussis* intracellular survival in macrophages and macrophage apoptosis (Valdez et al. 2016; Hewlett et al. 2006; Ahmad et al. 2016). In parallel, the oligomerized conformation of CyaA generates a cation-selective pore that induces potassium ion efflux from nucleated cells (Gray et al. 1998; Osickova et al. 1999). This activity promotes cell death caused by both apoptosis and necrosis (Basler et al. 2006; Khelef et al. 1993; Hewlett et al. 2006). In addition, CyaA-promoted potassium efflux induces IL-1 β production by dendritic cells via activation of caspase-1 and NALP3-containing inflammasome complex (Dunne et al. 2010) and activates mitogen-activated protein kinase (MAPK) and N-terminal protein kinase (JNK) signaling (Masin et al. 2015; Svedova et al. 2016). Hence, taken together CyaA uses both its AC domain and hemolysin moiety to induce cellular dysfunctions that promote bacterial survival and inhibit phagocyte-mediated bacterial clearance.

2.2.2 Effects of CyaA on *B. pertussis* colonization and host immune responses

—CyaA was characterized as a toxin in 1982, when it was shown that CyaA inhibited phagocytosis and oxidative burst by human neutrophils (Confer and Eaton 1982). Since that time, CyaA has been found to specifically target myeloid phagocytic cells, using CD11b/CD18 integrin (known as $\alpha_M\beta_2$ integrin or complement receptor 3, CR3) on the host cell surface as a receptor (Guermonprez et al. 2001). CyaA can also penetrate lipid bilayers in the absence of this receptor (Martin et al. 2004; Szabo et al. 1994) and intoxicate most cells but with reduced efficacy (Gordon et al. 1989; Gray et al. 1999; Eby et al. 2010; Bassinet et al. 2000; Hanski and Farfel 1985). In vivo, CyaA acts primarily on phagocytic cells to inhibit clearance and promote *B. pertussis* colonization (Gueirard et al. 1998; Harvill et al. 1999). However, CyaA-induced apoptotic cell death of bronchopulmonary cells is also described following administration of airway-isolated bacteria (Gueirard et al. 1998).

A role for CyaA in bacterial colonization was first described by Weiss et al. (Weiss et al. 1983). In that study, *B. pertussis* mutants not expressing CyaA were found to be non-lethal in a histamine-sensitizing in vivo mouse assay (Weiss et al. 1983). Further to this, expression of CyaA was found to be required for colonization and lethality of *B. pertussis* in infant mice (Goodwin and Weiss 1990; Weiss and Goodwin 1989). Both AC activity and the hemolysin moiety have been shown to be required for competent colonization and virulence by *B. pertussis* (Gross et al. 1992; Khelef et al. 1992). Indeed, in a later study using *B. pertussis* strains that express CyaA deficient in AC or hemolysin activity, it was determined that actions performed by the AC domain were required for bacterial colonization and persistence in mouse lungs, whereas the hemolysin moiety was not involved in colonization (Skopova et al. 2017). However, the hemolysin moiety did contribute to *B. pertussis*-induced lethality, penetration of *B. pertussis* across the epithelial lining and recruitment of myeloid phagocytic cells into *B. pertussis*-infected tissue (Skopova et al. 2017). Hence, the pore-

forming ability of CyaA is not required for bacterial persistence but contributes to *B. pertussis*-associated pathology.

2.3 CyaA as a pertussis vaccine component

Whole-cell formulations of pertussis vaccines (wP) displayed AC activity (Hewlett et al. 1977) and mass spectrometry analysis of wP detected the presence of CyaA (Boehm et al. 2018). However, the aP does not contain CyaA antigens. In studies using monoclonal antibodies and serum from convalescent individuals, it was found that antibodies against CyaA promote *B. pertussis* phagocytosis by neutrophils, validating one potentially beneficial effect of including a CyaA antigen in an aPV (Weingart and Weiss 2000; Weingart et al. 2000; Mobberley-Schuman et al. 2003). In addition, CyaA antibody-mediated neutrophil clearance of *B. pertussis* was shown to be important in an immune mouse challenge model (Andreasen and Carbonetti 2009). Given the potential for CyaA-mediated toxicity, current studies on developing CyaA as a vaccine antigen have focused on delineating the minimal essential regions of CyaA that confer protective immunity. Studies by Wang et al. show that the RTX domain of CyaA was sufficient to generate neutralizing antibodies and may represent an alternative to the use of full length CyaA (Wang et al. 2015). Indeed, in a mouse model, inclusion of the RTX domain in aP resulted in enhanced bacterial clearance after *B. pertussis* challenge, increased production of anti-PT antibodies, decreased production of proinflammatory cytokines and decreased recruitment of total macrophages (Boehm et al. 2018).

CyaA also displays an adjuvant effect in mice during immunization, with AC enzymatically inactive-CyaA generating greater and more potent adaptive immune responses than active CyaA (Orr et al. 2007; Cheung et al. 2006). When used as an adjuvant, CyaA induces the generation of antigen-specific Th17 cells by a pore-forming dependent mechanism (Dunne et al. 2010). Th17 cells mediate protective immunity to *B. pertussis* (Ross et al. 2013), hence generation of vaccine antigens that include specific functional regions of CyaA may prove beneficial in an acellular vaccine formulation.

3. TRACHEAL CYTOTOXIN

3.1 Background

Tracheal cytotoxin (TCT) is a peptidoglycan (PGN) fragment released by *B. pertussis*. PGN recycling is a process utilized by bacteria during cell division. First PGN is converted into its constituent parts, which are then available to the bacteria to be utilized in the synthesis of more PGN or for use as an energy source (Uehara and Park 2008). Bacteria producing PGN molecules containing diaminopimelic acid (DAP), or DAP-type PGNs, such as *Bacillus subtilis*, *Neisseria gonorrhoeae*, *Lactobacillus acidophilus* and *B. pertussis*, lose large amounts (25–50%) of the DAP from their cell wall to their growth media compared to *E. coli* (6%) (Mauck et al. 1971; Boothby et al. 1973; Goodell 1985; Goodell et al. 1978; Chaloupka and Strnadova 1972; Hebel and Young 1976). The inner membrane permease AmpG is a key component in this process (Jacobs et al. 1994). In *Bordetella* however, this transporter is defective, resulting in accumulation of extracellular fragments of PGN (Cookson et al. 1989a; Rosenthal et al. 1987; Mielcarek et al. 2006). Over 95% of these

PGN fragments released from *Bordetella* are of the structure N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-L-alanine-D-glutamyl-mesoDAP-D-alanine. This released monomeric fragment of PGN constitutes the virulence factor TCT. TCT is a 921 dalton disaccharide tetrapeptide PGN fragment. The anhydrous nature of the acetylmuramyl saccharide indicates that TCT is not just an accidentally released PGN fragment (which would contain reducing as opposed to anhydrous muramic acid residues) (Goldman and Cookson 1988). This finding led to the hypothesis that TCT is processed via an as yet unidentified murein transglycosylase.

3.2 TCT in pathogenesis

Host pattern recognition receptors trigger innate responses to PGN fragments. Following its release from the bacterial cell wall, non-ciliated cells of the airway epithelium internalize TCT (Flak and Goldman, 2001, (Flak et al. 2000; Flak and Goldman 1999). TCT is trafficked to the cytosol via PGN transporter Slc46A2 (Paik et al. 2017) where it is recognized by the pattern recognition receptor Nod1 (nucleotide-binding oligomerization domain protein 1) (Magalhaes et al. 2005). This leads to the activation of downstream NF- κ B signaling (Paik et al. 2017) and IL-1 α production by non-ciliated cells of the murine airway epithelium, in a synergistic manner with LPS (Flak et al. 2000). Interestingly, neither TCT nor *Bordetella* lipooligosaccharide (LOS) elicit strong IL-1 α production alone, but the combination of the two is a potent producer (Flak et al. 2000). Downstream effects of this activity include the production of nitric oxide (NO), which diffuses to neighboring ciliated cells causing cell death (Flak and Goldman 1999). In addition to promoting NO and IL-1 α mediated inflammation, TCT inhibits neutrophil chemotaxis (Cundell et al. 1994) preventing optimal immune responses.

In *Drosophila*, TCT triggers the activation of the IMD pathway following cytosolic recognition by PGN recognition protein PGRP-LE (Lim et al. 2006). Interestingly, mammalian PGN recognition protein 4 (PGLYRP4) has a protective role against TCT-mediated pathogenesis (Skerry et al. 2019). Hypersecretion of TCT, using mutant strains lacking PGN recycling molecule AmpG, or deficiency in PGLYRP4 causes increased early lung pathology in mice, suggesting that PGLYRP4 limits early TCT-induced inflammation (Skerry et al. 2019).

Each member of the pathogenic *Bordetellae* produce a chemically identical TCT molecule (Cookson et al. 1989b; Folkenin et al. 1987; Gentry-Weeks et al. 1988; Goldman and Cookson 1988). Additionally, all of the pathogenic *Bordetellae* induce a similar respiratory pathology typified by the destruction of the ciliated cells of the upper airways, in hosts ranging from humans (Paddock et al. 2008) to dogs (Oskouizadeh 2011; Bemis 1992) and turkeys (Arp and Fagerland 1987) and TCT is the only factor capable of replicating this ciliated cell extrusion that is the hallmark of the bordetelloses (Cookson et al. 1989a; Endoh et al. 1986; Goldman et al. 1982; Goldman and Cookson 1988).

TCT analogues in other bacteria are associated with similar pathologies, e.g. *Neisseria gonorrhoeae*-released PGN fragments have been associated with damage to the mucosa of human fallopian tubes (Melly et al. 1984). PGN monomers released from *Vibrio fischeri*

induce the regression of ciliated fields of *Euprymna scolopes* (Doino and McFall-Ngai 1995; Montgomery and McFall-Ngai 1994).

3.3 TCT in vaccination

The transition from the use of the whole cell to acellular pertussis vaccine has been associated with a reduction in the duration of immunity (Chen and He 2017). The whole cell vaccine, derived from intact *B. pertussis* organisms, likely contained TCT, even if only in the form of intact PGN. The acellular vaccine consists of recombinant subunit proteins of *B. pertussis* but does not contain TCT or PGN. Freund's complete adjuvant, a potent contributor to vaccine responses, contains muramyl peptides like TCT (Kotani et al. 1986). It has therefore been speculated that the loss of TCT in acellular vaccine formulations contributed to their waning immunogenicity (Goldman and Cookson 1988). Candidate live attenuated *B. pertussis* vaccine BPZE1 utilizes the *E. coli* PGN recycling machinery, AmpG, resulting in <1% TCT activity (Mielcarek et al. 2006). However, it contains the full complement of *Bordetella* PGN and therefore the potential adjuvanticity. Loss of PT, TCT and dermonecrotic toxin (DNT) resulted in a vaccine strain which successfully colonized hosts and elicited a protective immune response, without the associated pathophysiology of "wild-type" infection (Skerry and Mahon 2011; Skerry et al. 2009; Feunou et al. 2010).

TCT is identical in structure to slow wave sleep promoting factor FSu (Martin et al. 1984). In rabbits, FSu has been shown to induce excess slow-wave sleep following intraventricular infusion of picomolar concentrations (Krueger et al. 1982). The sleep-promoting effects of this factor are separate from its immunomodulatory potential (Krueger and Karnovsky 1987). These somnogenic qualities, along with the small size of TCT, led to the untested hypothesis that TCT may have been the reason behind the whole-cell vaccine-associated drowsiness (Goldman and Cookson 1988).

CONCLUSION

As we describe here, much of the pathogenesis of pertussis is attributable to the major secreted toxins, PT, CyaA and TCT, produced during *B. pertussis* infection (their activities and effects are summarized in Figure 1 and Table 1). Although there is still much to be learned about their activities and roles, it is clear that these toxins collectively suppress protective immune responses while exacerbating damaging pathologies in the respiratory tract and (in the case of PT) other organ systems. Further understanding of these toxin activities and the affected host responses will be important in the development of optimal therapeutics and vaccines to treat and prevent pertussis.

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Figure 1.
Summary of pertussis toxin (PT), adenylate cyclase toxin (CyaA) and tracheal cytotoxin (TCT) activities on cells.

Table 1.Effects and activities of the major *Bordetella pertussis* toxins.

Agent	Effect	Reference
Pertussis Toxin	ADP-Ribosylation of Gi/a class GPCRs	Katada <i>et al.</i> 2012
	Induction of leukocytosis	Morse and Morse 1976, Munoz <i>et al.</i> 1981, Hinds <i>et al.</i> 1996, Nogimori <i>et al.</i> 1984
	Inhibition of chemokine signaling	Pham <i>et al.</i> 2008, Beck <i>et al.</i> 2014
	Cardiac issues	Grimm <i>et al.</i> 1998, Wainford <i>et al.</i> 2008, Adamson <i>et al.</i> 1993, Zheng <i>et al.</i> 2005
	Reduction of vascular barrier integrity	Dudek <i>et al.</i> 2007
	Histamine sensitization	Munoz <i>et al.</i> 1981
	Insulinemia/hypoglycemia	Yajima <i>et al.</i> 1978
	Prevention of cough cessation	Maher <i>et al.</i> 2011
	Promotes colonization	Carbonetti <i>et al.</i> 2003, Connelly <i>et al.</i> 2012
	Inhibits early defense	Carbonetti <i>et al.</i> 2003, Carbonetti <i>et al.</i> 2005, Kirimanjeswara <i>et al.</i> 2008,
	Inhibits macrophage antibacterial responses	Andreasen and Carbonetti 2008
	Promotes lung inflammation	Carbonetti <i>et al.</i> 2007 Connelly <i>et al.</i> 2012, Khelef <i>et al.</i> 1994, Andreasen <i>et al.</i> 2009
Adenylate cyclase toxin	Hemolytic pore formation	Osickova <i>et al.</i> 1999, Fisher <i>et al.</i> 2012
	cAMP intoxication	Wolff <i>et al.</i> 1980, Hanski and Furfel 1985, Guo <i>et al.</i> 2005
	Inhibition of oxidative burst and phagocytosis	Confer and Eaton <i>et al.</i> 1982, Pearson <i>et al.</i> 1987, Kamanova <i>et al.</i> 2008, Friedman <i>et al.</i> 1987, Cerny <i>et al.</i> 2015
	Suppression of pro-inflammatory cytokines and chemokines	Boyd <i>et al.</i> 2005, Njakembo <i>et al.</i> 2000, Ross <i>et al.</i> 2004
	Inhibition of NET formation	Eby <i>et al.</i> 2014
	Induction of potassium ion efflux	Gray <i>et al.</i> 1998, Osickova <i>et al.</i> 1999
	Promotes cell death	Basler <i>et al.</i> 2006, Khelef <i>et al.</i> 1993, Hewlett <i>et al.</i> 2006
	Activation of MAP and JNK signaling	Masin <i>et al.</i> 2015, Svedova <i>et al.</i> 2016
	Promotes Colonization	Weiss <i>et al.</i> 1989
	Inhibits clearance	Gueirard <i>et al.</i> 1998
Tracheal Cytotoxin	Stimulation of Nod1	Magalhaes <i>et al.</i> 2005
	Nf-kB activation	Paik <i>et al.</i> 2017
	IL-1a production	Flak <i>et al.</i> 2000
	Ciliated cell death	Flak and Goldman 1999
	Inhibition of neutrophil chemotaxis	Cundell <i>et al.</i> 1994