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Bordetella adenylate cyclase toxin: a swift saboteur of host defense

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Bordetella that infect mammals produce a multifunctional repeat in toxin (RTX) adenylate cyclase toxin known as CyaA, an excellent example of bacterial sophistication in subverting host defense. Recent reports show that interaction of CyaA with tracheal epithelial cells aids adhesion of *Bordetella* to ciliated mucosa and induces production of the pro-inflammatory cytokine interleukin, IL-6. Myeloid phagocytes, attracted to the site of infection are the target of freshly secreted CyaA that binds to the $\alpha_M\beta_2$ integrin (CD11b/CD18), penetrates cells and promptly suppresses their bactericidal functions by converting cellular ATP to cAMP. Such uncontrolled cAMP signaling can also drive CD11b-expressing immature dendritic cells into a semi-mature state, possibly hijacking them to shape the local adaptive immune response towards tolerance of the pathogen.

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Introduction

Three *Bordetella* species are able to infect the respiratory tract of mammals: *B. pertussis*, a strictly human pathogen, causes pertussis (whooping cough) and remains endemic despite extensive vaccination programs [1,2]; *B. parapertussis* causes a somewhat milder form of pertussis in humans and can also infect sheep, whereas *B. bronchiseptica* establishes persistent and often asymptomatic respiratory infections in a wide range of mammals and occasionally also in immunocompromised humans. *B. pertussis* and *B. parapertussis* appear to have evolved from a *B. bronchiseptica*-like ancestor, essentially by losing genes and accumulating insertional mutations [3,4•]. Nevertheless, the three species express overlapping arrays of adhesins and toxins. All three species produce the adenylate cyclase toxin, CyaA, a dermonecrotic toxin (DNT) and a tracheal cytotoxin, TCT, whereas the type III secretion system (TTSS) effectors are only produced by *B. bronchiseptica* and the ovine *B. parapertussis* strains. By contrast, pertussis toxin (PT) appears to be produced exclusively by *B. pertussis*. Action of the *Bordetella* toxins, as well as the biology of *Bordetella* in general, have recently been covered in several comprehensive reviews [5–7].

In this review, we cover recent findings related to the interaction of CyaA with host cells. We focus on activation of CyaA by calmodulin, the importance of proximity between bacteria and cell in toxin action, the interaction of the toxin with cellular receptor, and immunomodulatory consequences of toxin action on dendritic and epithelial cells. For references to earlier studies on action of CyaA, refer to a previous review by Ladant and Ullman [8].

CyaA is a unique fusion of a cytolysin with a calmodulin-activated adenylate cyclase enzyme

The adenylate cyclase toxin of *Bordetella*, which is also a hemolysin and cytolysin, referred to in the literature as CyaA, ACT, AC-Hly or AC toxin, is a member of the RTX (repeat in toxin) family of bacterial pore-forming toxins [9]. CyaA differs, however, from the other members of this family, however, by being a protein fusion of a cell-invasive and highly potent adenylate cyclase (AC) enzyme with a typical RTX cytolysin moiety (Figure 1). The N-terminal AC domain (~400 residues) penetrates the cytosol of target cells, where the AC is activated by binding of eukaryotic calmodulin and catalyzes unregulated conversion of cellular ATP to cAMP, a key second messenger signaling molecule [8]. The RTX hemolytic moiety (~1300 residues) accounts for binding of the toxin to target cells, for translocation of the AC domain into the cell cytosol, directly through the cytoplasmic membrane, and for making the cell membrane permeable by cation-selective toxin pores [8]. CyaA, thus, combines two distinct strategies employed by bacterial toxins for the manipulation of host cell physiology: interference with cellular signaling pathways by elevating cAMP level; and interference with ion homeostasis through disruption of the permeability barrier of the cellular membrane.

Upon activation by cytosolic calmodulin, the AC enzyme of CyaA acquires a particularly high catalytic potency, k_{cat} ~2000 s^{-1}, in conversion of ATP to cAMP [10]. Recently, Guo *et al* [11••] solved the crystal structure of the adenylate cyclase domain of CyaA in complex with the C-terminal fragment of calmodulin (C-CaM) (Figure 1)

Glossary

Adaptive immunity: Response of antigen-specific lymphocytes to pathogen-derived antigens. Adaptive immunity (also known as acquired immunity) depends on presentation of the antigen by antigen-presenting cells (APCs), in particular dendritic cells (DCs), and the subsequent activation of T and B lymphocytes.

Dendritic cell (DC): Immature DCs are phagocytic cells that circulate, or reside under resting conditions in tissues such as mucosa. Upon maturation induced by microbial and inflammatory products, DCs function as antigen-presenting cells (APCs) that migrate into mucosa-associated lymph nodes and initiate adaptive immune response. Fully mature DCs are characterized by up-regulation of MHC II and of co-stimulatory molecules, such as CD80, CD86 and CD40, and by secretion of pro-inflammatory cytokines.

Innate immunity: Host response that acts as the first line of defense. Innate immunity is omnipresent and involves a variety of resistance mechanisms, such as opsono-phagocytosis and oxidative burst, with broad spectrum activity on microorganisms, including bactericidal activity of phagocytic cells.

Regulatory T cells (Tr): T lymphocytes that control adaptive immunity. For example, Tr cells can fully suppress immune responses or modify the cellular immune responses (Th1) or antibody responses (Th2).

RTX (repeat in toxin): Important virulence factors produced by a wide range of Gram-negative bacteria. RTX toxins are unconventional pore-forming toxins that are characterized by binding of calcium ions to numerous sites within glycine and aspartate-rich repeats harboring a conserved sequence motif GGXGXD. The produced inactive protoxins acquire biological activity upon covalent posttranslational fatty-acylation on ε-amino groups of one or two conserved internal lysine residues that is accomplished by co-expressed protein toxin acyltransferases.

Semi-mature dendritic cell (smDC): An intermediary differentiation stage of DCs between immature and mature DCs. As with mature DCs, semi-mature DCs also express high amounts of surface markers such as MHC II and co-stimulatory molecules, but do not secrete pro-inflammatory cytokines. *In vivo,* semi-mature DCs are actively tolerogenic by inducing IL-10-secreting regulatory $CD4^+$ T cells (Tr) in an antigen-specific manner.

Type I secretion system: RTX protein secretion machinery used by Gram-negative bacteria for secretion of toxins, proteases, lipases and other RTX proteins. Such factors pass directly from cytoplasm across both the inner and outer bacterial membranes in a single step, without a periplasmic intermediate, employing a 'channel-tunnel' assembly spanning the membranes and the periplasmic space.

Type III secretion system: A bacterial 'molecular syringe' injector assembly which enables the translocation of effector proteins directly from the site of production within cytoplasm of the adhering bacterial cells into the cytoplasm of colonized eukaryotic target cells. It involves about 20 components that assemble into a large structure that spans both bacterial membranes and penetrates also the host cell membrane.

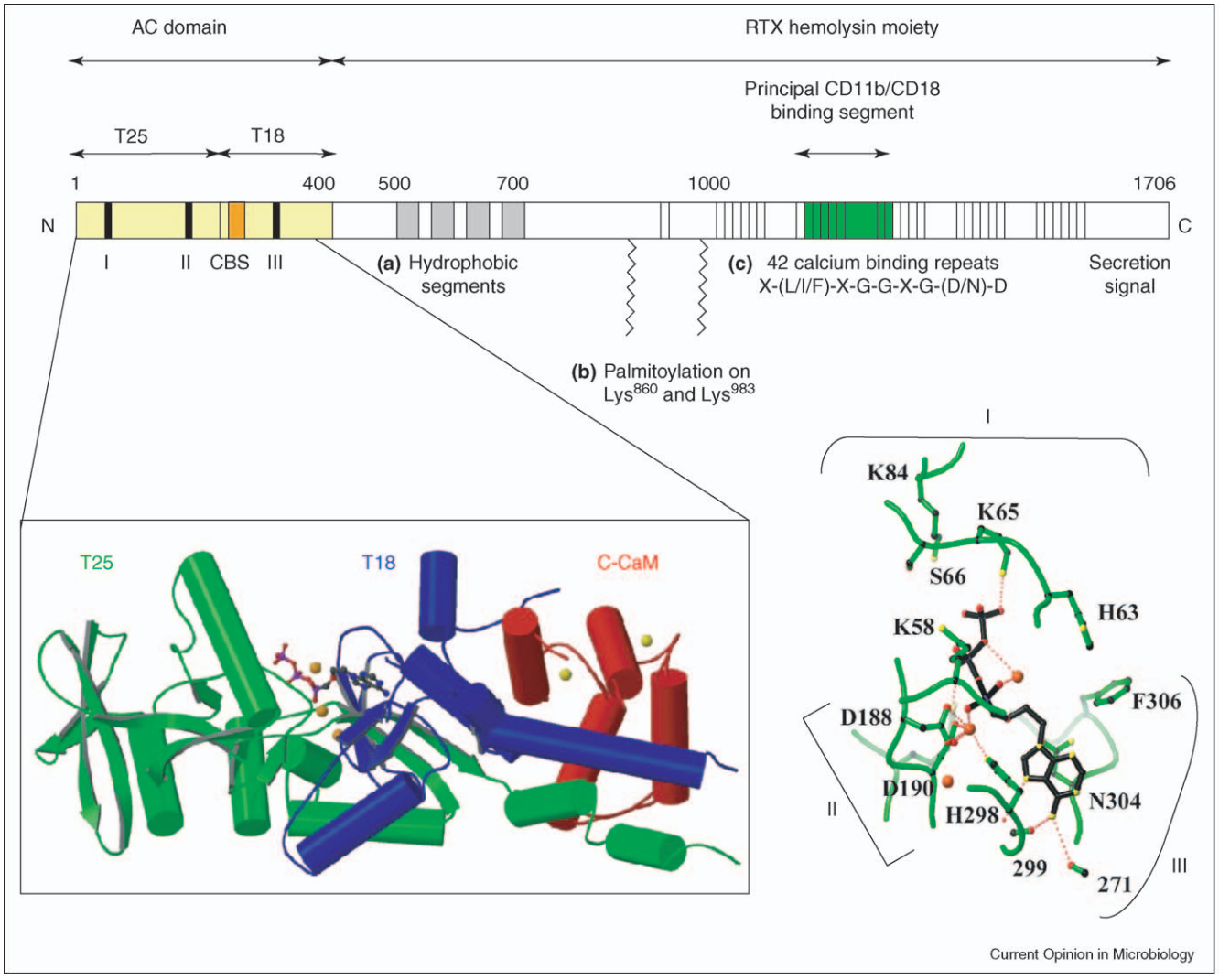
and performed a detailed analysis of the catalytic mechanism employed by AC. This provided essential structural and mechanistic clues for understanding the high catalytic efficiency of AC ($\sim 10^7\ M^{-1}\ s^{-1}$) and enabled definitive identification of catalytic residues. The structure reveals that four discrete regions of the prokaryotic AC bind to calcium-loaded eukaryotic calmodulin with a large, buried contact surface, where a tryptophan residue (W242) of AC plays a crucial role and makes extensive contacts with the, calcium-induced, hydrophobic pocket of calmodulin [11••].

Interestingly, AC affinity for calmodulin and AC–calmodulin interactions differ substantially from those of edema factor (EF), another calmodulin dependent adenylate cyclase toxin, produced by *Bacillus anthracis*, which, otherwise, has quite similar catalytic properties to those of the AC of CyaA [10,12]. The affinity of AC to calmodulin is ~100-fold higher than that of EF to calmodulin, and the need for this high affinity might reflect the different routes of entry and cellular localization of both toxins [11••]. Whereas the AC domain of CyaA is directly translocated across the plasma membrane into the cytosol [13•] and probably stays close to the plasma membrane, the EF enters the cytosol upon endocytosis and translocates across the endosomal membrane [14]. Therefore, AC and EF can be expected to compete with different sets of calmodulin-binding proteins [11••]. In contrast to the delay in action of EF, the direct translocation of the AC domain of CyaA across the plasma membrane and rapid activation by calmodulin, enables immediate synthesis of large amounts of cAMP that rapidly paralyze $CD11b^+$ target cells upon exposure to CyaA.

CyaA is a short-lived toxin acting in the proximity of the bacteria

CyaA is secreted as an unfolded protein, through a type I 'channel-tunnel' secretion system spanning the Gram-negative bacterial cell envelope, directly from Bordetella cytoplasm into the external environment [15]. Most of the CyaA, however, remains associated with the bacterial surface, as shown initially for *B. pertussis* by Hewlett *et al* [16]. It was, therefore, hypothesized that surface-bound CyaA is the active form of the toxin and that association of CyaA with filamentous hemagglutinin on the bacterial cell surface enabled delivery of CyaA from the bacterium to the target cell [17]. A recent study by Gray *et al* [18••], however, established that only the freshly secreted, newly synthesized toxin molecules, and not the bacterial-surface-associated CyaA, are able to penetrate target cells and elicit elevation of cAMP levels. These authors demonstrated that proximity, but not direct physical interaction between the bacterial and the target cell membranes, was important for efficient intoxication of host cells by CyaA. Secretion of CyaA from the bacteria does not appear to occur in a polarized manner or to be limited to the bacterium–target cell interface but, rather, occurs in all directions, hence, it was proposed that freshly secreted CyaA proteins form an 'atmosphere' of active toxin molecules around the bacterial cell (Figure 2) [18••]. Some of the newly synthesized CyaA molecules are secreted near the target cell surface and can reach cellular receptors and penetrate target cells before aggregating and losing activity (Figure 2). This model is supported by a recent observation that, in contrast to pertussis toxin, CyaA does not act as a conventional soluble factor *in vivo* and the intranasal administration of purified CyaA does not increase the colonization capacity of a *B. pertussis* strain defective in production of CyaA in mice [19]. The requirement of close association of bacteria with its target cell for

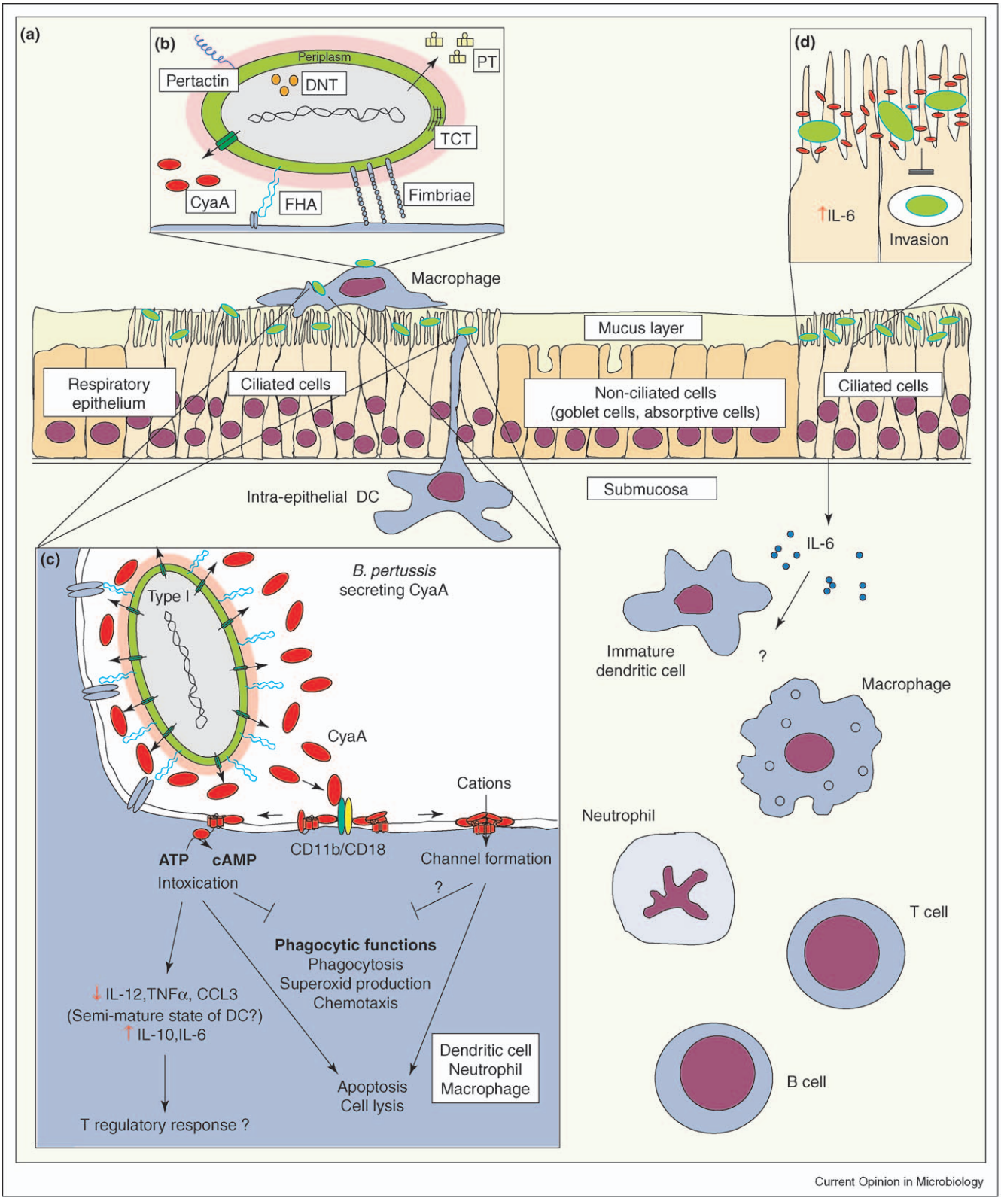
Figure 1



Structural organization of the 1706 residue-long *Bordetella pertussis* adenylate cyclase (CyaA) toxin molecule. The N-terminal catalytic domain of ~400 residues is a cell-invasive and calmodulin-activated adenylate cyclase (AC) enzyme; T25 and T18 correspond to subdomains of AC, CBS represents the main calmodulin-binding site, and boxes I, II and III highlight the AC segments involved in catalysis. The catalytic domain is enlarged to show the recently solved three-dimensional structure of the ternary complex of AC with the C-terminal fragment of calmodulin and adefovir diphosphate, a metabolite of an anti-viral drug that tightly binds into the catalytic site of CyaA thereby mimicking binding of ATP [11••]. Structure of the catalytic site with bound adefovir diphosphate (black structure) is given in further close-up (right). It shows that aspartates 188 and 190 (D188 and D190), as well as histidine 298 (H298) of AC, are crucial for binding of the catalytic Mg^{2+} metal ions (red balls) and asparagine 304 (N304) is involved in positioning of ribose, arginine 37 and three lysine residues, K58, K65 and K84 are involved in binding the triphosphate of the ATP substrate of AC. Deprotonation of 3' OH of ATP is accomplished by histidine 63 (H63), the central catalytic residue of AC. The last ~1300 residues represent an RTX hemolysin moiety (Hly) of CyaA. This harbors **(a)** the hydrophobic domain forming cation-selective membrane pores, **(b)** the acylated domain, where post-translational activation of the protoxin is accomplished through CyaC-mediated covalent fatty-acylation of either of ε-amino groups of Lys^{860} and Lys^{983} [27•] and **(c)** the RTX repeat blocks binding of calcium ions (~40 Ca^{2+} per CyaA molecule) and allowing interaction of toxin with its β_2 integrin receptor CD11b/CD18 on myeloid phagocytic cells [25]. Concerted action of these segments allows toxin interaction with and translocation of the AC domain across target cell membrane.

effective cell intoxication by CyaA to occur, indeed agrees with observations from the early *in vitro* studies, showing that CyaA readily forms inactive toxin aggregates in solution [20]. Upon secretion, a significant proportion of CyaA molecules appear to become trapped on the bacterial surface, and are unable to penetrate target cells [18••]. Interestingly, this bacterial cell-associated CyaA still appears to be of potential benefit to the producing bacteria, as Edwards *et al* [21•] showed that CyaA associated with surface of *B. bronchiseptica* cells might make an important contribution to the ability of the bacteria adhere to ciliated epithelium.

Figure 2



Schematic depiction of key steps of CyaA interaction with target cells in the course of *Bordetella pertussis* infection of the host respiratory tract mucosa **(a)** *B. pertussis* colonizes the ciliated columnar epithelium in the trachea and nasopharynx and can also adhere to host immune cells (e.g. incoming macrophages or resident intraepithelial dendritic cells). **(b)** Main virulence factors of *B. pertussis*. Adhesins: filamentous

CyaA targets CD11b-expressing myeloid cells

CyaA promiscuously binds to and detectably raises cAMP levels in several cell types *in vitro* [8]. *In vivo* studies, however, indicate that CyaA exerts its activity primarily on myeloid phagocytes, such as alveolar macrophages and neutrophils [22,23]. CyaA was found to bind with high affinity to the $\alpha_M\beta_2$ integrin, CD11b/CD18 (also known as CR3 or Mac-1) expressed on myeloid cells, such as macrophages, neutrophils and dendritic cells, and on natural killer cells [24]. The main receptor-interacting domain of CyaA is located within the glycine and aspartate-rich repeat region (Figure 1), and calcium binding and post-translational acylation of CyaA were both shown to be required for a tight and productive interaction of CyaA with cells expressing the CD11b/CD18 receptor [25]. Although acylation of lysine residue 983 (Lys^{983}) of CyaA is both necessary and sufficient for toxin activity on $CD11b^-$ cells [26], covalent palmitoylation of either of the ε-amino groups of the internal residues Lys^{860} and Lys^{983} enables CyaA to tightly engage the CD11b/CD18 receptor and exert toxin activity on $CD11b^+$ cells [27•].

Immunomodulatory effects of CyaA activity on dendritic cells

Aside from inhibiting the bactericidal activities of phagocytic cells by suppressing superoxide production, chemotaxis or phagocytosis [28–32], CyaA might play an important pathophysiological role in *Bordetella* infections by acting on dendritic cells and affecting secretion of cytokines, thereby modulating the polarity of induced T-lymphocyte responses (Figure 2). Bagley *et al.* [33•] demonstrated that, *in vitro*, the cAMP-elevating activity of CyaA promoted increased expression of the MHC class II and co-stimulatory molecules, CD80, CD83 and CD86, in human monocyte-derived dendritic cells and also suppresses production of the pro-inflammatory cytokines interleukin (IL)-12 and tumor necrosis factor (TNF)-α. It does this through cAMP-regulated protein kinase A-dependent signaling pathways. Subsequent studies confirmed that cAMP-elevating activity of CyaA influences maturation of murine bone marrow-derived dendritic cells (BMDCs) and up-modulates lipopolysaccharide-induced production of the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10, while simultaneously inhibiting pro-inflammatory lipopolysaccharide-driven TNF-α, IL-12 and CCL3 chemokine production by BMDCs and macrophages [34•,35•]. In a recent study with live *B. bronchiseptica* bacteria, Skinner *et al* [36••] demonstrated that CyaA cooperates with effectors injected into the cells by the TTSS (see glossary), driving murine BMDCs into a semi-mature state. This results in upregulated production of the anti-inflammatory cytokine IL-10 and in decreased production of the pro-inflammatory cytokines IL-12 and TNF-α. Semi-mature dendritic cells, unable to secrete pro-inflammatory cytokines appear, indeed, to promote the expansion of regulatory T (Tr) cells [37]. Hence, manipulation of the maturation status of dendritic cells (see glossary) by the concerted action of CyaA and TTSS effectors might promote tolerogenic immune responses that would enable persistent colonization of the host respiratory tract by *B. bronchiseptica* [36••]. Altogether, the current data suggest that CyaA has potential to alter host immune responses through cAMP-elevation in $CD11b^+$ dendritic cells, and that this toxin activity might be of particular importance during *Bordetella* infections of the respiratory epithelium, where intra-epithelial dendritic cells reside (Figure 2). Interaction of CyaA with dendritic cells, hence, deserves detailed examination.

Potential effects of CyaA on epithelial cells

Many reports indicate that *Bordetella* not only adhere to but can also invade epithelial cells [38], however, the cell invasion appears to be followed by efficient killing of the bacteria [38,39•], and so the biological significance of epithelial cell invasion by *Bordetella* spp remains unclear. *B. pertussis* mutants deficient in CyaA were previously found to be more invasive into human tracheal epithelial cells than the wildtype, and so it was hypothesized that expression of CyaA could be an anti-invasive mechanism (Figure 2), enabling *B. pertussis* to avoid destruction within tracheal epithelial cells [38]. More importantly, CyaA action appears to account for induction of pro-inflammatory cytokine IL-6 production in human tracheal epithelial cells (Figure 2) in response to colonization by *Bordetella* [40••]. The biological consequences of epithelial cell stimulation by CyaA (i.e. of indirect modulation of immune cells through cytokine production by epithelial cells) have yet to be investigated.

Conclusions

In the past five years, significant progress has been made in understanding the interaction of CyaA with target cells.

Figure 2 Legend (*continued*) hemagglutinin (FHA), fimbriae and pertactin. Toxins: pertussis toxin (PT), adenylate cyclase toxin (CyaA), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT). **(c)** Enlarged section schematically depicts the reported effects of CyaA action on macrophages, neutrophils and dendritic cells. Upon CyaA secretion by the Type I secretion system, an atmosphere of toxin molecules forms around bacteria. Most of the produced toxin molecules remain attached on bacterial surface (pink border), as a result of the interaction with filamentous hemagglutinin (blue hairpin), while some toxin molecules are released into the bacterial surroundings and are capable of binding host cells. CyaA binds myeloid phagocytes through their $\alpha_M\beta_2$ integrin (CD11b/CD18) receptor molecule, and, upon insertion into membrane, the toxin can translocate its AC domain into the cytosol and catalyze formation of cAMP and/or form cation-selective pores in the cell membrane and perturb cellular ion homeostasis. These activities can individually, or in synergy, influence the various cellular signal transduction cascades, modulate or paralyze cellular bactericidal and antigen-presenting functions, or induce apoptotic cell death and lysis. **(d)** Enlarged section of CyaA interaction with ciliated epithelial cells. CyaA action accounts for induction of production of the pro-inflammatory cytokine IL-6 by tracheal epithelial cells upon interaction with *B. pertussis* and for inhibition of bacterial uptake into epithelial cells.

The most important new insights are; description of a specific protein receptor (CD11b/CD18) for CyaA on myeloid phagocytic cells, determination of the AC domain structure in complex with calmodulin, and the finding that only newly synthesized and secreted toxin and not the bacterial-cell-associated CyaA, is effective in elevating intracellular cAMP concentrations in $CD11b^+$ target cells. An important new focus of research on CyaA is the significant potential of the toxin to exert immunomodulatory activity through action on $CD11b^+$ dendritic cells, and by inducing secretion of the pro-inflammatory cytokine IL-6 from tracheal epithelial cells. Discovery of the physiological consequences of this modulatory action of CyaA on immune and epithelial cells holds the promise of yielding novel insights into the cross-talk of *Bordetella* with host tissues.

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Using transmission electron microscopy and invasion and persistence assays, the authors demonstrate that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are rapidly degraded upon uptake by human tracheal epithelial cells.

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This study revealed that CyaA accounts for the capacity of *B. pertussis* to induce production of high levels of the pro-inflammatory cytokine IL-6 by human epithelial cells.