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Dissertation

Bordetella Adenylate Cyclase: Molecular mechanism of Action and Its Use for Antigen Delivery

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I hereby declare that I have elaborated this thesis independently, and all the resources employed as well as co-authors are indicated. I further declare that I did not submit this thesis, or an essential part of it, to obtain other, or the same university degree.

Prague, 1st November, 2009

Signature

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INDEX

SUMMARY

The first part of this PhD. thesis deals with molecular mechanism of action of the adenylate cyclase toxin (CyaA), a key virulence factor of the whooping cough agent *Bordetella pertussis*. CyaA belongs to the family of RTX (Repeat-in-ToXin) proteins secreted by Gram-negative bacteria and primarily targets myeloid phagocytes, expressing the CD11b/CD18 integrin receptor (also known as $\alpha_M\beta_2$, CR3 or Mac-1). Upon binding, CyaA permeabilizes cell membranes by forming small cation-selective pores, and subverts cellular signaling by delivering into host cells an adenylate cyclase (AC) enzyme that converts ATP to cAMP. Elevation of the cytosolic cAMP levels by CyaA then knocks down bactericidal functions of host innate immunity.

CyaA is unique among other enzymatically active toxins in its capacity to penetrate cells directly from cell surface across the cytoplasmic membrane, without the need for endocytosis. Penetrating activity of CyaA depends on plasma membrane potential and on an intact, acylated and calcium-loaded RTX cytolysin moiety. By examining a set of 18 CyaA constructs that bear overlapping deletions within AC domain and a $CD8^+$ OVA T-cell epitope tag, we showed that the first 371 amino-terminal residues are dispensable for the CyaA capacity to deliver a passenger OVA epitope into cytosol of dendritic cells, as determined *in vitro* by stimulation of OVA-specific $CD8^+$ T cells. This observation suggested a passive passenger role of the AC domain during its membrane penetration.

In addition, we demonstrated that CyaA suppresses the bactericidal activities of macrophages by provoking futile membrane ruffling and showed for the first time that cAMP signaling of the CyaA toxin causes a rapid and complete inhibition of CR3-mediated phagocytosis. We further reported that the molecular mechanism of the repeatedly documented capacity of CyaA to undermine bactericidal activities of macrophages may well rely on RhoA inactivation, as a result of cAMP signaling. Besides that, by flow cytometry analysis and ELISA assays, we characterized the ability of CyaA to modulate maturation of dendritic cells (DCs), and showed that CyaA suppresses LPS-induced CD40 and CD54 molecule expression, and enhances IL-10 cytokine production. Moreover, we demonstrated that CyaA-treated DCs have a reduced capacity to prime proliferation of antigen-specific $CD4^+$ as well as $CD8^+$ T cells, and we unraveled the prominent subversive role of cAMP-activated PKA in these processes. Collectively, these findings corroborate the previous observations that CyaA subverts host immune responses.

The second part of the PhD. thesis is focused on the use of adenylate cyclase toxoids for antigen delivery. The penetration of recombinant CyaA/AC$^-$ toxoids to cell cytosol could be previously exploited for delivery of passenger CD8$^+$ epitopes to the major histocompatibility complex (MHC) class I presentation pathway, and induction of cytotoxic CD8$^+$ T lymphocyte (CTL) responses. An efficient therapeutic antitumor immunity in mice, and a full prophylactic protection against lethal lymphocytic choriomeningitis virus challenge was, indeed, conferred upon immunization with CyaA/AC$^-$ toxoids that bear appropriate CD8$^+$ T-cell epitopes. We tested here the capacity of the CyaA-CSP toxoids, containing an epitope of the circumsporozoite protein of the rodent malaria parasite *Plasmodium berghei,* to induce protective anti-malaria immunity in mice. Immunization of mice with CyaA-CSP toxoid induced high numbers of CSP-specific IFN-γ-secreting CD8$^+$ T cells, while no protective immunity against challenge with *P. berghei* sporozoites was achieved. However, when the anti-CTLA-4 was administered during boost immunization, or when CyaA-CSP toxoid was employed in a heterologous prime/boost vaccination regimen, using live recombinant *Salmonella* delivering the CSP epitope through type III secretion system as a primary vaccination strategy, we observed significant enhancement of the CSP-specific CD8$^+$ T cells and induction of protective immunity. Taken together, these results document the potential of CyaA to confer protection against a parasitic infection and to boost efficacy of vaccines in heterologous prime/boost immunizations.

ABBREVIATIONS

AC	adenylate cyclase
APC	professional antigen-presenting cell
CR3	complement receptor 3
CSP	circumsporozoite protein
CTL	cytotoxic $CD8^+$ T lymphocyte
CyaA	adenylate cyclase toxin
CyaA/AC^-	adenylate cyclase toxoid
DC	dendritic cell
DNT	dermonecrotic toxin
Epac	guanine exchange protein directly activated by cAMP
FcR	Fc receptor
FHA	filamentous hemagglutinin
Hly	hemolysin
ICAM-1	intracellular adhesion molecule-1, CD54
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PKA	protein kinase A
PTX	pertussis toxin
RBC	red blood cell
ROS	reactive oxygen species
RTX	Repeat-in-ToXin
TCT	tracheal cytotoxin
Treg cells	T regulatory cells
TTSS	type III secretion system

INTRODUCTION

Immune system of the airways

The airways serve as a piping system for gas transport from the outside atmosphere to the lung respiratory zone of alveoli, where the respiration (the exchange of oxygen and carbon dioxide with the blood) occurs. The daily inhalation of 10,000 litres of air leads to an exposition of human respiratory system to a variety of airborne pathogens. The observation that respiratory infections are nevertheless rare argues for the presence of an efficient host defense system at the mucosal surfaces of the airways and lungs (Bals and Hiemstra, 2004).

The mammalian immune system is comprised of two branches, the innate immune system and the adaptive immune system, which work in tandem to provide resistance to infection. The innate immune response constitutes the first line of host dense and is responsible for immediate recognition and control of microbial invasion. The adaptive immune response, on the other hand, is responsible for elimination of pathogens in the late phase of infection and for generation of immunological memory (Mogensen, 2009).

Airways, respiratory epithelium and airway epithelial cells

Airways are defined anatomically as the upper respiratory tract, which includes the nasal passages, paranasal sinuses and pharynx, and the lower respiratory tract that begins at the larynx, continues to the trachea and divides into the two main bronchi entering the roots of the lungs (Figure 1A).

Respiratory epithelium which lines the luminal surfaces of the airways is positioned at the interface with environment, being the primary site for the introduction and deposition of the potentially pathogenic microorganism into the body. The maintenance and regulation of the immune responses to the microorganisms is therefore mediated by the respiratory epithelium (Diamond *et al.*, 2000). The epithelium is classified as ciliated pseudostratified columnar epithelium and consists of ciliated, goblet and basal cells. The cells are arranged in a single layer of cells, that make contact with the basement membrane (basal lamina). The epithelium, however, creates the illusion as though several layers of cells would be present, which led to the designation “pseudostratified” (Figure 1B). The epithelium is covered by the airway mucus layer. The inhaled material trapped in the mucus is removed by the co-ordinated beating of the cilia of epithelial cells towards the throat, where it is either

expectorated, or swallowed to the stomach (Diamond *et al.*, 2000).

In addition to their mucociliary clearance function, epithelial cells are known to secrete several families of molecules that are involved in the protection against infection by bacteria, viruses and fungi (Figure 2). Antimicrobial products secreted by epithelial cells include, (i) enzymes: lysozyme, phospholipases, peroxidases, complement components; (ii) permeabilizing peptides: defensins, cathelicidins, bacterial permeability-increasing protein; (iii) opsonins: complement components, collectins, pentraxins; (iv) protease inhibitors: secretory leukocyte proteinase inhibitor, elafin; (v) toxic small molecules: reactive oxygen species (ROS), thiocyanate, nitric oxide; (vi) binding/neutralizing proteins: mucins, serum amyloid A, lactoferin; and others. Production of many of these substances by epithelial cells is initiated upon stimulation of cellular pathogen-recognition receptors, such as Toll-like receptors, NOD-like receptors and RNA helicases. These receptors recognize highly conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) and initiate anti-bacterial response (Diamond *et al.*, 2000; Kato and Schleimer, 2007; Schleimer *et al.*, 2007).

Airway epithelial cells upon activation by bacterial PAMPs and/or inflammatory cytokines also recruit inflammatory cells to the airways to combat the infection via release of chemoatractants (Figure 2A and 3A), (Kato and Schleimer, 2007; Schleimer *et al.*, 2007; Thompson *et al.*, 1995). When primary cultures of bovine airway epithelial cells were

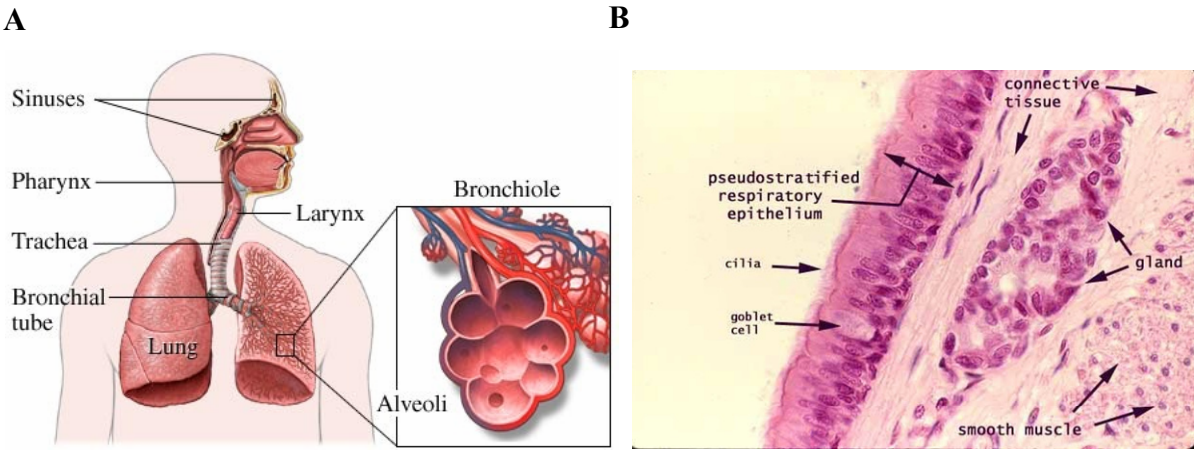

Fig. 1. Respiratory system and airways. A) The human respiratory tract can be subdivided into 3 segments, the upper respiratory tract, the lower respiratory tract and the lungs. The upper respiratory tract includes the nasal passages, paranasal sinuses and pharynx (throat), whereas lower respiratory tract consist of larynx (voice box), trachea and bronchi. Finally, lungs comprise respiratory bronchioles and alveolar ducts with alveoli and alveolar sacs; from http://nursingcrib.com/wp-content/uploads/upper-respiratory-system.jpg; **B)** Airways are lined by the ciliated pseudostratified columnar epithelium in which ciliated, goblet and basal cells are present. The ciliated cells are columnar epithelial cells with ciliary modification, whereas goblet cells are columnar epithelial cells producing airway mucus. The basal cells are small cuboidal cells thought to differentiate in other cell types in case of epithelium injury, thereby restoring the healthy epithelial cell layer; from http://media.photobucket.com/image/respiratory%20epithelium/lovesthesunset/anatomy%20and%20physiology/LRrespiratoryepitheliumlabeled.jpg.

stimulated with LPS from *E. coli,* production of neutrophil chemoattractants was observed (Koyama *et al.*, 1991). In addition to neutrophils, other inflammatory cells recruited to the airways include lymphocytes, monocytes and eosinophils (Diamond *et al.*, 2000). Interestingly, during airway infection/inflammation, epithelial cells up-regulate surface expression of ICAM-1 (intracellular adhesion molecule-1), the ligand for the leukocyte integrin CD11a/CD18. This integrin is expressed on the surface of neutrophils, monocytes, lymphocytes and eosinophils (Diamond *et al.*, 2000; Thompson *et al.*, 1995). Epithelial cells, however, in addition to recruiting and stimulating inflammatory cells may participate in the down-regulation of inflammation by production of several families of anti-inflammatory molecules, including cytokines (IL-10, TGF-β), soluble cytokine receptors/receptor antagonists (sIL-1RN, sIL-13RA2, sTNFR1) and prostaglandin PGE_2 (Kato and Schleimer, 2007; Thompson *et al.*, 1995), thus maintaining the balance between inflammatory and anti-inflammatory processes.

Besides that, epithelial cells interact with intraepithelial and subepithelial DCs, and alter the ability of DCs to activate and polarize naive T cells (Schleimer *et al.*, 2007), thus

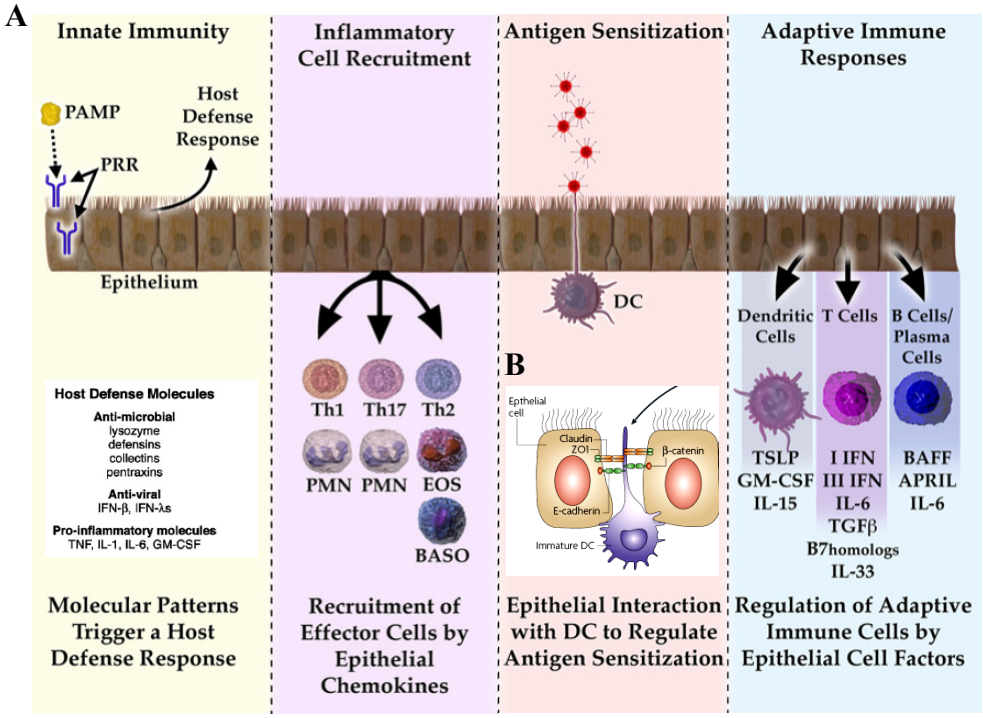

Fig. 2. Role of airway epithelial cells in innate and adaptive immune responses. A) Epithelial cells express pattern-recognition receptors (PRR) and release anti-microbial products into the airways. During inflammatory and immune responses, epithelial cells release specific chemokines that recruit subsets of granulocytes (*PMN*, polymorphonuclear leukocyte; *EOS*, eosinophil; *BASO*, basophil) and T cells that are appropriate to the particular immune response. Epithelial cells also interact with interepithelial and subepithelial DCs to alter the ability of DCs to activate and polarize naive T cells. Finally, epithelial cells regulate the adaptive immune response by expression of soluble and cell-surface molecules that alter the function of DCs, T cells, and B cells in the airways (Kato and Schleimer, 2007; Schleimer *et al.*, 2007); **B)** DCs sample the airway lumen by forming dendritic extensions between epithelial cells. DCs form tight junctions with epithelial cells through their expression of occludin (not shown) and claudin family proteins, as well as zonula occludens 1 (ZO1); (Hammad and Lambrecht, 2008).

modulating adaptive immune responses (Figure 2). They also boost induction of adaptive immune responses under inflammatory/infection conditions, as they recruit DCs to the airways. In addition, their secretory products and cell surface molecules alter the adaptive immune responses by modulating the functions of T cells, and B cells, present in the airways (Kato and Schleimer, 2007; Schleimer *et al.*, 2007).

Airway phagocytes

In healthy individuals, the predominant cell type recovered in induced sputum samples which primarily contains cells from the surfaces of bronchial airways, are the macrophages, followed by neutrophils (Belda *et al.*, 2000). These cells due to their surface location represent one of the first lines of cellular defense against inhaled pathogens (Alexis *et al.*, 2006). Macrophages and neutrophils are professional phagocytes. They are characterized by expression of a set of phagocytic receptors designed to recognize, bind to and trigger internalization of foreign objects, including pathogens. Different phagocytic receptors can be involved in particle recognition, depending on whether or not opsonins are involved in the phagocytic process. Opsonin-dependent phagocytosis involves either Fc receptors (mainly FcγR) or complement receptors (CR1, CR3 and CR4), which bind particles that have either IgG or complement bound to their surface, respectively. Opsonin-independent phagocytosis is triggered by engagement of cellular receptors, such as mannose or type A scavenger receptors which recognize molecular motifs directly on the surface of pathogens. Following uptake, internalized particles are destroyed as they progress along the degradative endocytic pathway. This relies on production of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), acidification (H^+) of phagosomes, recruitment of bactericidal peptides and enzymes like proteases and hydrolases into phagosomes, and others (Celli and Finlay, 2002; Rosenberger and Finlay, 2003). Indeed, sputum phagocytes are functional with respect to their phagocytic capacity, oxidative burst generation and expression of cell-surface receptors associated with innate host defense (Alexis *et al.*, 2000).

In response to pathogens and/or inflammatory cytokines, resident professional phagocytes in the airways, similarly to airway epithelial cells, recruit other immune cells to the site of infection and influence the nature of the immune response through production of various chemokines and/or cytokines (Figure 3A), (Rosenberger and Finlay, 2003; Thompson *et al.*, 1995).

Airway dendritic cells

DCs mediate airway antigen capture, transport and presentation to naive T cells. DCs of the airways are situated below the basement membrane in an immature state and can extend their dendrites between epithelial cells directly into the airway lumen. This "periscope" function is constitutively active and provides a mechanism for continuous immune surveillance. At least in the mouse lungs, intraepithelial $CD103^+$ DCs express the tight-junction proteins claudin-1, claudin-7 and zonula-2, which form tight junctions with airway epithelial cells, thereby explaining how DCs can sample the content of the lumen without disturbing the functions of the epithelium barrier (Figure 2B), (Hammad and Lambrecht, 2008; Sung *et al.*, 2006). There are between 500 and 1000 DCs per mm^2 of the airway in most species. However, airway DCs become more numerous in response to pathogens and/or proinflammatory cytokines (Upham and Stumbles, 2003). Indeed, epithelial cells upon stimulation with TNF, IL-1 and bacterial PAMPs induce DC migration into epithelium via production of CCL20 (Kato and Schleimer, 2007).

After stimulation by PAMPs and antigen capture, DCs from airways migrate to draining lymph nodes and acquire ability to prime naive T cells (Figure 3B). This process involves up-regulation of surface expression of MHC class II glycoproteins and costimulatory

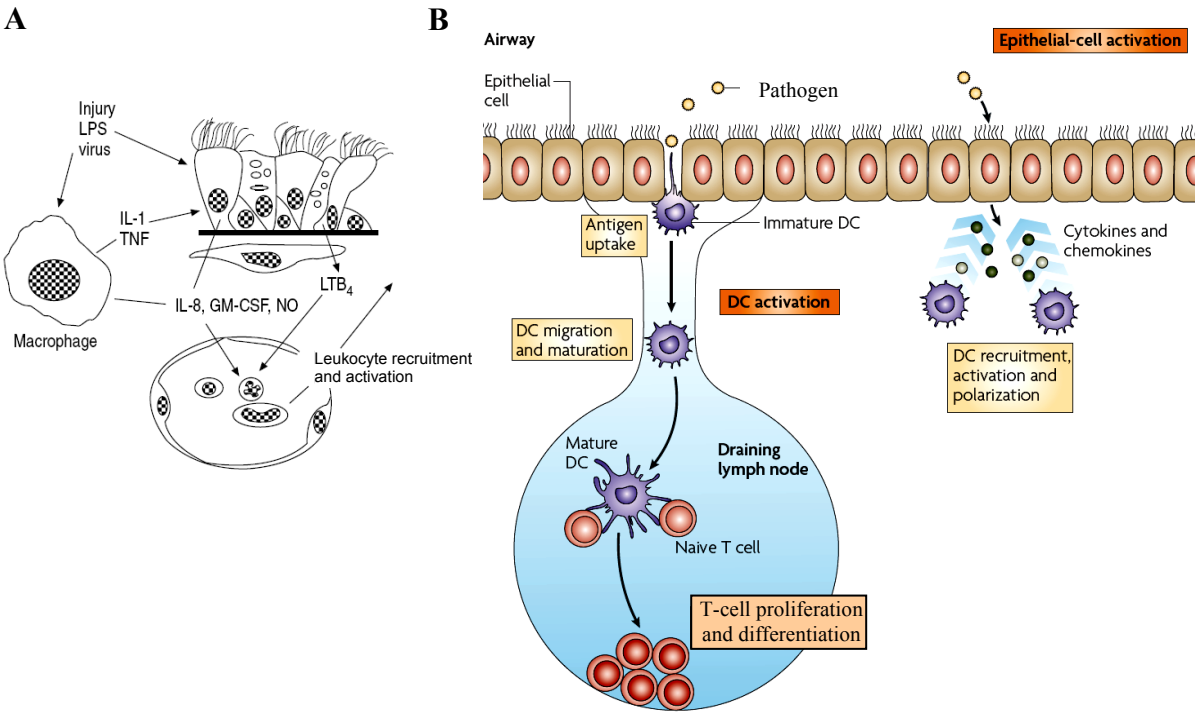

Fig. 3. Schematic representation of the activated respiratory epithelium. A) Inflammatory stimuli, e.g. LPS or viral infection, initiate release of mediators including LTB4, IL-8, NO, and GM-CSF from epithelial cells, and IL-1 and TNF-α from macrophages. These mediators further augment the inflammatory response by stimulating epithelial cells, and recruiting and activating other leukocytes (Thompson *et al.*, 1995); **B)** DCs within the respiratory epithelium, are the cells that integrate signals from inhaled pathogens and the elicited innate immune response into signals that direct cells of the adaptive immune system (Hammad and Lambrecht, 2008).

molecules (CD40, CD80, CD86) and changes in the expression of specific chemokine receptors. The tissue homing receptors for CCL5, CCL11 and CCL20 are down-regulated whereas lymph node homing receptor CCR7 which responds to CCL19 and CCL21 is up-regulated (Hammad and Lambrecht, 2008; Upham and Stumbles, 2003).

DCs, however, do not react in a stereotypic fashion to all pathogens but respond to different classes of microorganisms with different patterns of cytokine synthesis and/or expression of costimulatory and adhesion molecules. DCs might also adopt different phenotypes depending on the contact with modulatory tissue factors. Importantly, depending on their phenotype, DCs might regulate T cell polarization into Th1-, Th2-, Th17- and regulatory T-cell subsets (Kapsenberg, 2003; Zhou *et al.*, 2009). From the soluble factors secreted by DCs, IL-12 is a key cytokine responsible for initiating and maintaining INF-γ-producing Th1 cells. IL-10, on the other hand, can inhibit IL-12 synthesis and Th1 differentiation. In some models, the IL-10 produced by lung DCs appears to be critical for Th2 responses to inhaled antigens (Hammad and Lambrecht, 2008; Upham and Stumbles, 2003).

Further, IL-6 produced by APCs induces IL-4 production by naive T cells and their differentiation into Th2 effector cells while inhibiting Th1. Similarly, PGE_2 is another molecule produced by DCs that is known to favor Th2 development and inhibit Th1 differentiation (Upham and Stumbles, 2003). Recent studies have also shown that upon treatment with thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine molecule produced by airway epithelial cells, DCs induce naive $CD4^+$ T cells to differentiate into an inflammatory Th2 cell phenotype of asthmatic airways (Hammad and Lambrecht, 2008; Ito *et al.*, 2005).

In contrast to T cell priming by pathogen-activated DCs, exposure to inhaled harmless antigens under normal conditions induces a state of immunological tolerance. In this regard it is well established that soluble antigens delivered via mucosal surfaces, in the absence of inflammatory signals, induce a profound state of T cell tolerance. One possible explanation is that, harmless antigens cannot fully activate the DCs, but induce only DC partial activation. The partially mature DCs would then induce an abortive immune response, either by activating T cells that fail to reach the threshold required for prolonged survival and are finally deleted, or by inducing IL-10 and TGFβ-producing T regulatory (Treg) cells (Hammad and Lambrecht, 2008).

T cells in the airway epithelia

T cell migration into airways and shape of T cell effector response is controlled by the airway epithelial cells. Epithelial cells upon stimulation with the Th1 cytokine INF-γ, type I or type III interferons, up-regulate production of CXCL9, CXCL10 and CXCL1 chemokines, that are ligands for CXCR3. The CXCR3 chemokine receptor is expressed on activated T cells, predominantly of the Th1 phenotype, as well as on NK cells and a subset of circulating memory $CD4^+$ and $CD8^+$ T cells. This suggests the presence of a feed-forward mechanism in which IFN-γ released by Th1 cells in the epithelium induces production of more CXCR3 ligands, thus amplifying the Th1-type immune response. A similar mechanism regulates Th2 responses. Epithelial cells in response to Th2 cytokines, IL-4 and IL-13 up-regulate production of chemokines attracting Th2 cells, namely, CCL1 and CCL17 (Kato and Schleimer, 2007; Schleimer *et al.*, 2007).

Airway epithelia cells were recently shown to express also PD-L1 and PD-L2, that are ligands for PD-1, an inhibitory molecule expressed on activated T cells. The strong expression of PD-L1 and PD-L2 molecules in activated epithelium *in vivo* may reflect part of a feed-back inhibitory mechanism (Kim *et al.*, 2005). Epithelial cells also express Fas ligand, which can limit lymphocyte survival and CD40, which can stimulate epithelial production of RANTES, MCP-1, IL-8 and ICAM-1 (Kato and Schleimer, 2007; Schleimer *et al.*, 2007).

B cells in the airway epithelia

Production of IgA and IgE in the upper airways has been proposed to be important for both, protection against pathogens and also in the pathogenesis of airway allergic diseases. The IgA and IgE are produced by B cells in the airway mucosa. Upon production, the polymeric forms of these immunoglobulins are transported by epithelial cells via the polymeric Ig receptor to the airways (Kato and Schleimer, 2007; Schleimer *et al.*, 2007).

The genus *Bordetellae*

Bordetellae are small, Gram-negative coccobacilli that are closely related to the environmental bacteria and some opportunistic pathogens of genera *Achromobacter* and *Alcaligenes*. The genus *Bordetellae* comprises eight species, namely, *B. pertussis, B. bronchiseptica, B. parapertussis*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum*, and *B. petrii* (Mattoo and Cherry, 2005).

B. bronchiseptica cluster

B. pertussis, B. bronchiseptica and *B. parapertussis* colonize the respiratory tract of mammals, but their host range and severity of the disease they cause differ. *B. pertussis* is strictly a human pathogen and it is the causative agent of an acute respiratory disease known as pertussis, or whooping cough. *B. bronchiseptica* has a broad mammalian host range, causing chronic and often asymptomatic respiratory infections in a wide range of animals and occasionally in humans. Finally, *B. parapertussis* is divided into two phylogenetically distinct populations, which are genetically diverse. Human-adapted *B. parapertussis*$_{hu}$ is responsible for pertussis-like disease, while ovine-adapted *B. parapertussis*$_{ov}$ causes chronic infection of the sheep respiratory tract. There appears to be little or no transmission between the sheep and human reservoirs of *B. parapertussis* (Mattoo and Cherry, 2005).

B. pertussis, B. bronchiseptica and *B. parapertussis* strains are, due to their very close phylogenetic relationship, collectively referred as "*B. bronchiseptica* cluster". Despite their varied host range, the principles underlying the pathogenesis are similar. They are acquired through infected droplets from other hosts and display a strong tropism for the cilia of the respiratory mucosa, which are the main site of infection (Mattoo and Cherry, 2005). The virulence of these tree *Bordetella* strains is controlled by the Bvg two-component system (Figure 4) that regulates the expression of highly similar virulence factors (Table 1), (Shrivastava and Miller, 2009). Although, the pertussis toxin is synthesized solely by *B. pertussis*, both *B. parapertussis* and *B. bronchiseptica* possess pertussis toxin genes without expressing them (Table 1).

Based on common molecular strain typing techniques, it was suggested that *B. pertussis*, human-adapted *B. parapertussis*$_{hu}$ and ovine-adapted *B. parapertussis*$_{ov}$ arose independently from a *B. bronchiseptica*-like ancestor. The independent evolution of *B. pertussis* and *B. parapertussis*$_{hu}$ from a *B. bronchiseptica*-like ancestor was further

supported by comparative genome sequencing of representative strains of *B. pertussis*, *B. parapertussis*$_{hu}$ and *B. bronchiseptica* (Cummings *et al.*, 2004; Mattoo and Cherry, 2005; Parkhill *et al.*, 2003).

The genomes of *B. pertussis* and *B. parapertussis* have a smaller size than the genome of *B. bronchiseptica* and compared to *B. bronchiseptica* genome, exhibit a considerable increase in the number of IS elements, accumulation of pseudogenes, and many chromosomal rearrangements. It seems likely that expansion of IS elements driving modifications and deletions of existing genes, and rearrangements of global regulatory sequences, rather than the gene acquisition, was important process in the evolution of *B. pertussis* and *B. parapertussis* from a *B. bronchiseptica*-like ancestor (Cummings *et al.*, 2004; Parkhill *et al.*, 2003; Preston *et al.*, 2004).

Adoption of a host-restricted life-style by *B. pertussis* and *B. parapertussis* may have relaxed the selection against chromosomal rearrangements. Chromosomal rearrangements and deletions, then in turn, may have driven the speciation of *B. pertussis* and *B. parapertussis* by altering regulatory networks, modifying metabolic pathways or eliminating antigenic proteins that made them susceptible to immune surveillance. Indeed, large-scale loss and inactivation

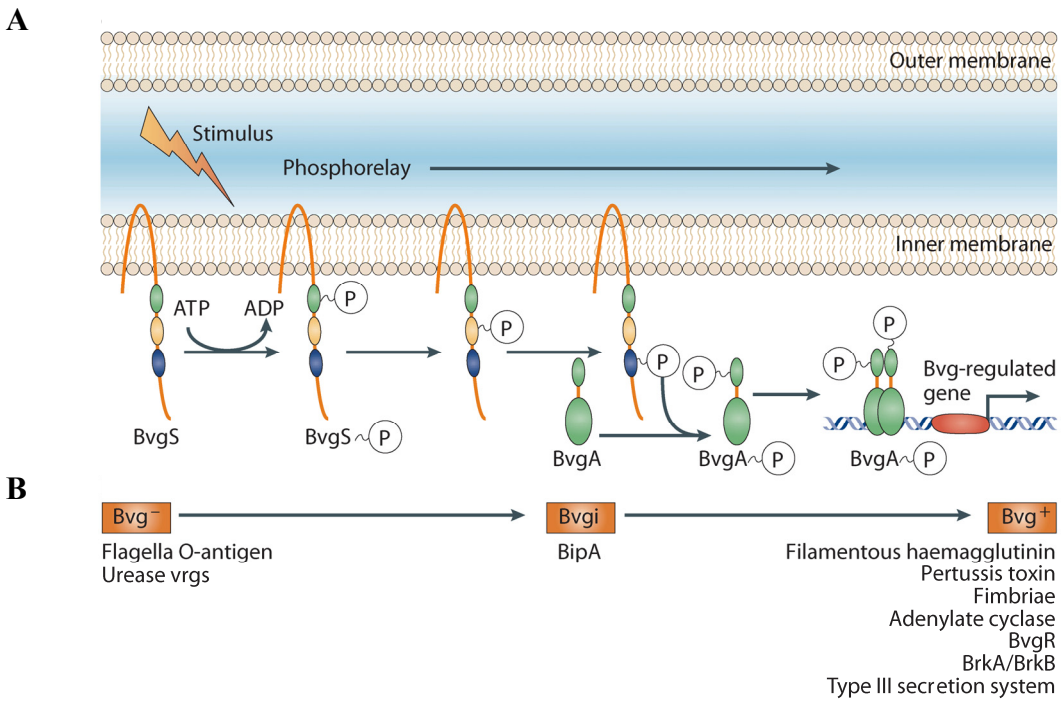

Fig. 4. The Bvg two-component system. A) Detection of a periplasmic stimulus activates BvgS and leads to the transfer of a phosphate group from a donor (probably ATP) to BvgS. The phosphate group is relayed along BvgS and ultimately to BvgA. Phosphorylated BvgA has transcriptional regulator activity and activates the transcription of several genes. This Bvg-active state is referred to as the Bvg$^+$ phase. When the system is silent, the bacteria adopt the Bvg phase that is characterized by the expression of a subset of Bvg phase-specific genes. Identification of an intermediate phase (Bvgi) that includes the expression of Bvgi-specific genes indicates that Bvg regulates a spectrum of responses; **B)** The figure lists factors that are expressed at each Bvg phase. Vrg, *vir*-repressed genes; (Preston *et al*., 2004).

of genes, together with IS element expansion appears to be similar with other pathogens that seem to have exploited a specific niche relatively recently. Signs of similar evolutionary processes are observed, for example in the genome sequences of *Salmonella typhi*, which is a human-restricted pathogen, in contrast to *S. enterica* serovar Typhimurium, which has a broader host range (Parkhill *et al.*, 2001). In common with *B. pertussis*, *S. typhi* has numerous pseudogenes and has lost several proteins that are thought to be involved in interactions with multiple hosts. It has also been proposed that the increase in the number of pseudogenes is caused by an increased fixation of mutations owing to accelerated genetic drift. This, in turn, is due to an evolutionary bottleneck that was caused by the occupation of a new niche by a small population of cells (Cummings *et al.*, 2004; Preston *et al.*, 2004).

Importantly, *B. pertussis* adaptation to humans by losing genetic material via IS elements is still in progress. Recent *B. pertussis* isolates have less genetic material as compared to those circulating some years ago. With the replacement of whole pertussis vaccine by subunit vaccines, containing only few selected bacterial antigens, one could envisage the emergence of strains of *B. pertussis*, which will not express the vaccine antigens, and thus may escape the vaccine-induced immunity (Bouchez *et al.*, 2008). Indeed, in France that is a country with high coverage of vaccination with subunit vaccines, such *B. pertussis* isolates have been already collected for the first time, in 2007 (Bouchez *et al.*, 2009).

Other *Bordetellae* species

Of the other *Bordetellae* described, *B. avium* is the causative agent of bordetellosis in avian species, which is similar to a respiratory disease. *B. hinzii* is found mainly as a commensal of respiratory tract of birds, but has a pathogenic potential in immunocompromised humans. *B. holmesii* has been associated with septicemia, however more recently; it was recovered also from nasopharyngeal specimens of patients with pertussis-like symptoms. *B. trematum* has been described as an occasional pathogen of humans, causing ear and wound infections but has never been associated with respiratory tract infections. Finally, *B. petrii* was isolated from the environment and is the only environmental species among the otherwise host-restricted and pathogenic members of the genus *Bordetellae* (Mattoo and Cherry, 2005; Sebaihia *et al.*, 2006).

TABLE 1. Secreted factors implicated in *Bordetella* virulence (Shrivastava and Miller, 2009).

Substrate / Homologies	Genome location /Secretion pathway	Function
Adenylate cyclase toxin (CyaA) /Member of RTX family of toxins	*cyaA* (encodes CyaA) and *cyaBDE* (encode T1SS) are proximal to one another / T1SS	Self-translocates across plasma membrane, catalyzes production of cAMP in host cells, essential virulence factor
Fimbriae /Type 1 and P pili in uropathogenic *E. coli*	*fim2*, *fim3* fimbrial subunit genes unlinked to *fimBCD* biogenesis genes / T2SS export fimbrial subunits to periplasm	Adherence, required for tracheal persistence *in vivo*; co-ordinates with FHA for colonization in mice
BteA /No homology to characterized proteins	*bteA* is unlinked to the *bsc* T3SS apparatus locus / T3SS	Required for cytotoxicity *in vitro* and persistence *in vivo*
Pertussis toxin (PT) /PT is a member of the ADP-ribosylating toxin family; Ptl T4SS components are homologous to VirB proteins in *Agrobacterium tumifaciens*	*ptxA-E* (encode PT subunits S1–S5) are adjacent to *ptlA-I* (encode T4SS); genes present but not expressed in *B. parapertussis* and *B. bronchiseptica* / T4SS	Protective antigen; facilitates colonization *in vivo*; inhibits agonist-induced GDP release from target G proteins to block agonist-induced responses
BrkA /C-terminal domain is homologous to pertactin and other autotransporters	Divergently transcribed to *brkB* (required for serum resistance activity) / Autotransporter (T5SS)	Resistance to serum killing; involved in adherence and invasion
Filamentous hemagglutinin (FHA) /Model for two-partner secretion in Gram-negative bacteria including: HMW proteins in *Haemophilus*; ShlA cytolysin in *Serratia marcescens*; CdiA from *E. coli*	*fhaB* (encodes FHA) located upstream of *fimBCD* and *fhaC*, which is required for secretion /Two-partner secretion (T5SS)	Major adhesin *in vitro*, important colonization factor *in vivo*; overcomes bacterial clearance by mucociliary escalator; enhances expression of ICAM-1 on target cells
Pertactin /Unique cargo domain, transport domain homologous to other autotransporters	Sole gene in operon /Autotransporter (T5SS)	Contributes to attachment to CHO and Hela cells *in vitro*, *in vivo* role uncertain
Tracheal colonization factor (TCF) /C-terminal domain is homologous to pertactin and other autotransporters	Sole gene in operon /Autotransporter (T5SS)	Required for colonization of mice
Vag8 /C-terminal domain is homologous to pertactin	Sole gene in operon /Autotransporter (T5SS)	Null mutants colonize mice as efficiently as wild type strains
BcfA /Homologous to BipA	Adjacent to putative dehydrogenase /Autotransporter-like	Required, along with BipA, for colonization of murine respiratory tract
BipA /Similar structure to intimins and invasins in enteric pathogens, homologous to BcfA	Sole gene in operon /Autotransporter-like	Bvg ‘intermediate phase’ gene; combinatorial role with BcfA in tracheal colonization
Unknown /Homologous to known T6SS structural components	Structural genes identified by homology are present in *B. bronchiseptica* and *B. parapertussis* /T6SS	Unknown
Dermonecrotic toxin (DNT) /Member of dermonecrosis-inducing bacterial toxin family; *E. coli* CNF-1 and CNF-2, *Y. pseudotuberculosis* CNF-γ	Sole gene in operon /Unknown, may be released following cell lysis	Activates small Rho family GTPases; causes skin lesions following injection; role in *B. bronchiseptica*-induced turbinate lesions
Tracheal cytotoxin /TCT is released during growth by *Neisseria* and *Vibrio* species	Release is due to insertion in *ampG* /Cell wall derived disaccharide tetrapeptide; released during growth	Induces cytopathology including loss of ciliary action and extrusion of epithelia cells; increases production of iNOS in host cells; activates Nod1 intracellularly

Bordetella pertussis, the agent of whooping cough

The whooping cough, or pertussis disease, has been clinically recognized since the sixteenth century and its causative agent, *Bordetella pertussis,* was first isolated by Bordet and Gengou in 1906. Pertussis is considered to be a disease of infants and young children, but adults may also be infected and develop a persistent cough (Birkebaek *et al.*, 1999; Gilberg *et al.*, 2002). The clinical course of whooping cough is divided into three stages: (i) the initial *catarrhal* stage marked by an irritating cough and slight fever, lasting 1–2 weeks, (ii) *paraoxysmal* cough characterized by an inspiratory 'whoop' as air rushes into the lungs against a narrowed glottis, which lasts 1–6 weeks, (iii) a lasting period of convalescence marked by decreased paroxysms, persistent cough and secondary bacterial infections. Severe consequences of this infection include encephalopathy, irreversible brain damage, pneumonia, lung collapse, increased intrathoracic pressure and haemorrhages due to ruptured small and large blood vessels (Babu *et al.*, 2001).

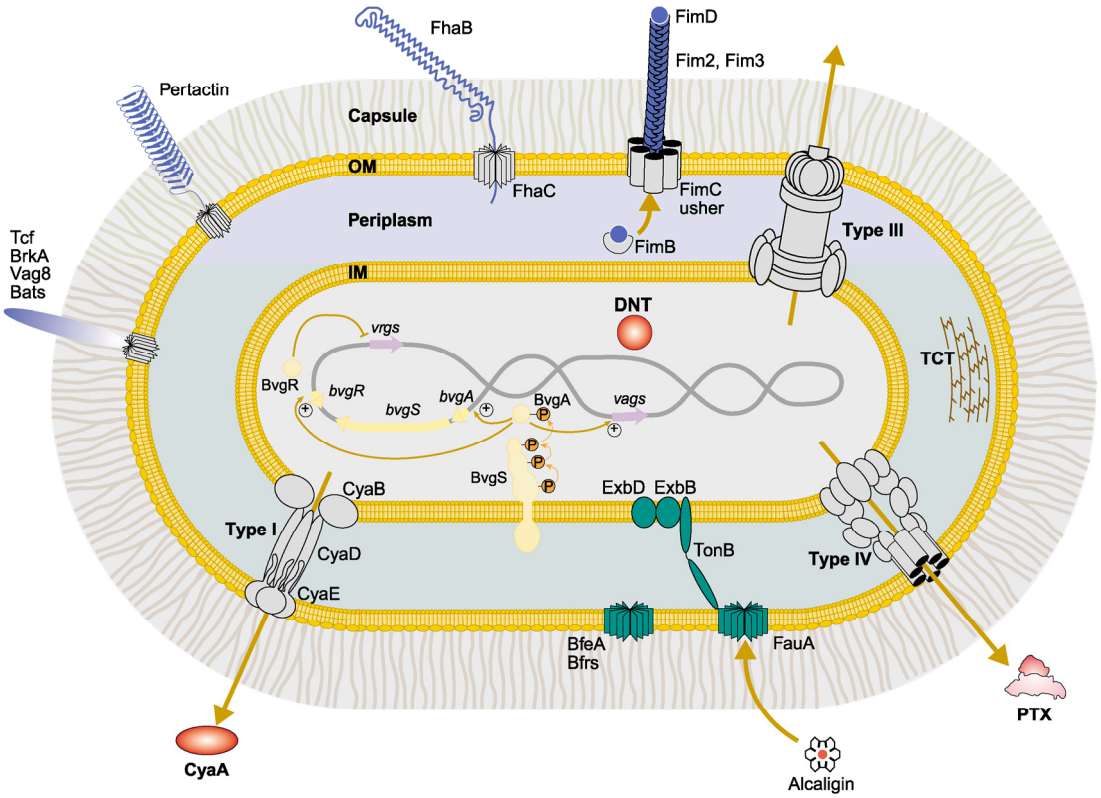

Fig. 5. *B. pertussis* virulence factors. *B. pertussis* is depicted as a Gram-negative organism with inner and outer membranes (IM and OM), a periplasm and a capsule. The adhesins Fim, FhaB, pertactin, Tcf, BrkA, Vag8 and Bats are shown in blue; the toxins PTX, CyaA and DNT are in red; the accessory proteins FhaC, FimB, FimC, Type III, Type IV and Type I are in grey; the iron uptake systems ExbB/ExbD, TonB, FauA, BfeA and Bfrs are in green; and the regulatory systems BvgA, BvgS and BvgR are in beige. The large brown arrows represent the orientation of export and import of virulence factors and siderophores, respectively. The thinner brown arrows show the phosphorelay and the regulation circuit; (Locht *et al.*, 2001).

The mechanisms by which *B. pertussis* causes the disease are still unknown, however the pathogenic potential of the *B. pertussis* relies on multiple virulence factors, which are under the control of a two-component transcriptional regulatory system Bvg (Figure 4). Some of the virulence factors participate in the adhesion of the bacteria to the respiratory tract of the host, such as fimbriae, filamentous hemagglutinin (FHA), pertactin. Others are protein toxins, responsible for the local and systemic effects, such as adenylate cyclase toxin (CyaA), pertussis toxin (PTX) and dermonecrotic toxin (DNT); (Locht *et al.*, 2001). Recently also a functional TTSS and its effector proteins were identified in *B. pertussis* (Fennelly *et al.*, 2008). In addition to that *B. pertussis* produces a non-protein toxin, the tracheal cytotoxin (TCT) responsible for damage of the ciliated epithelium (Figure 5).

Indeed, establishment of infection in the respiratory epithelia requires that bacteria are able to resist the clearance action of the mucociliary escalator as well as the killing effects of defensins, complement and other antimicrobial factors. Further, bacteria should overcome the protective anti-bacterial functions of airway phagocytes and also induction of adaptive humoral and T cell responses. In the murine model of infection by *B. pertussis*, evidence suggests that clearance of the bacteria depends on recruitment of neutrophils and macrophages to the lungs, local secretion of inflammatory cytokines, and the induction of *B. pertussis*-specific Th1 and Th17 cells, and antibody responses (Higgins *et al.*, 2006; Kirimanjeswara *et al.*, 2005; Mahon *et al.*, 1997; McGuirk and Mills, 2000; Mills *et al.*, 1993). In addition, IFN-γ production by NK cells was reported to prevent bacterial dissemination from the respiratory tract (Byrne *et al.*, 2004; Mahon *et al.*, 1997).

Various virulence factors produced by *B. pertussis* were reported to suppress and modulate the host immune response to *B. pertussis,* thus enabling bacterial persistence and contributing to the severe disease pathology (Figure 6). In addition to subversion of innate immune host response, *B. pertussis* infection also induces antigen-specific Th17 cells and IL-10-producing Treg cells (Fennelly *et al.*, 2008; McGuirk *et al.*, 2002). The Treg cells can subvert the development of adaptive immunity and delay clearance of bacteria from the lungs (McGuirk and Mills, 2002). The Th17 profile of the immune response to *B. pertussis* infection was on the other hand, proposed to mediate the chronic autoimmune reaction in the airways, and thus support the major pathology associated with *B. pertussis* infection — the cough (Carbonetti, 2007).

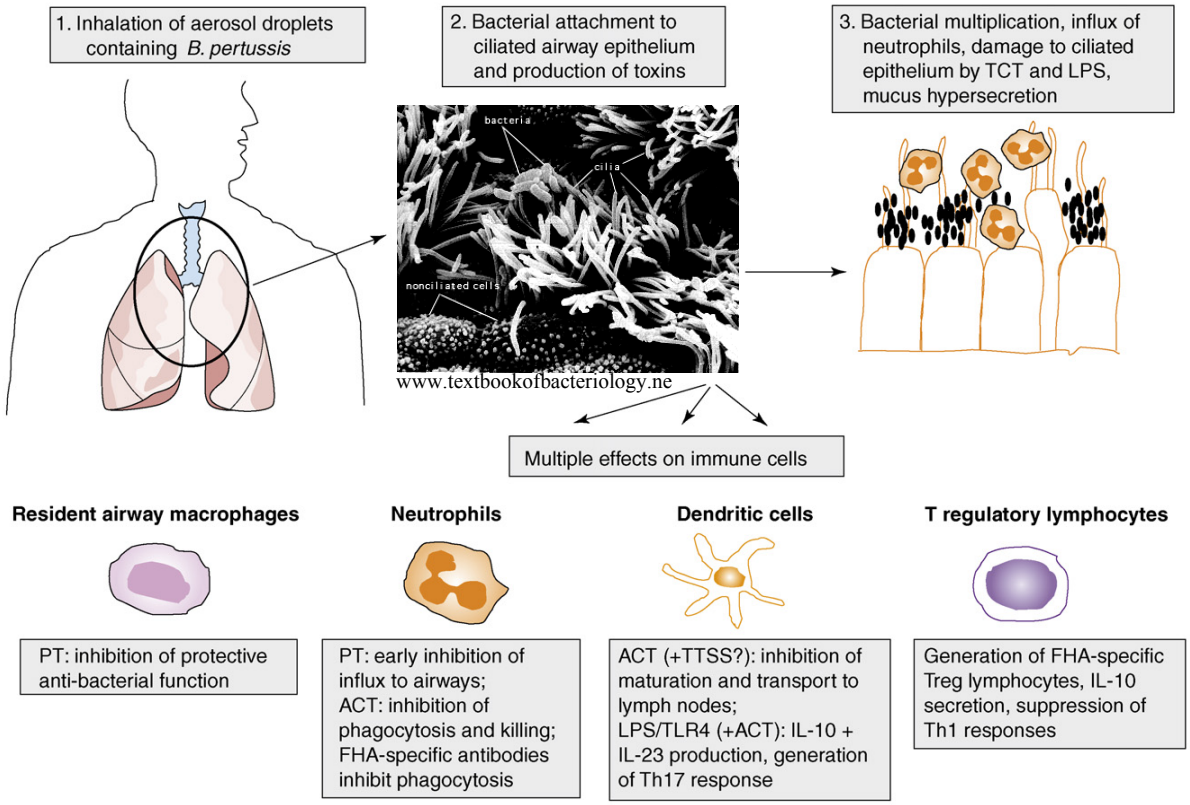

Fig. 6. Representation of respiratory tract infection by *B. pertussis* and its immunomodulatory effects. PT, pertussis toxin; ACT = CyaA, adenylate cyclase toxin; FHA, filamentous hemagglutinin, TTSS, type III secretion system; (Carbonetti, 2007).

Adenylate Cyclase Toxin of *Bordetella pertussis*

Adenylate cyclase toxin (known also as CyaA or ACT) is an important virulence factor common to all three species of the "*B. bronchiseptica* cluster", namely, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica.*

In 1984, Weiss and colleagues demonstrated that CyaA plays a key role in *Bordetella* virulence, by showing that *cyaA::Tn5* mutants, unable to produce the CyaA, exhibit a substantially higher LD_{50} in the infant mice model of intranasal *B. pertussis* infection than wild type strains (Weiss *et al.*, 1984). Since then CyaA was repeatedly reported to play a particularly important role in the early stages of *Bordetella* colonization of the host respiratory tract and to contribute to numerous pathological effects in the murine model of lung infection, such as efficient pulmonary colonization, induction of histopathological lesions in lungs and recruitment of inflammatory leukocytes (Goodwin and Weiss, 1990; Gross *et al.*, 1992; Gueirard *et al.*, 1998; Khelef *et al.*, 1994; Khelef *et al.*, 1992; Weiss and Goodwin, 1989)

CyaA is a bifunctional RTX toxin that delivers into host cells an adenylate cyclase (AC) enzyme, and permeabilizes cell membrane by forming small cation-selective pores. It was the first enzyme toxin shown to be capable to directly penetrate across the cytoplasmic membrane of target cells and to reach the cytosol without the need for endocytosis (Gordon *et al.*, 1988; Ladant and Ullmann, 1999; Schlecht *et al.*, 2004). With reduce efficacy the toxin can promiscuously bind and detectably raise cAMP levels in a variety of cell types, as was shown in numerous *in vitro* studies (Gao *et al.*, 2002; Gordon *et al.*, 1989). However, *in vivo* studies suggested that during murine respiratory infection with *B. pertussis* and *B. bronchiseptica* species, CyaA specifically targets phagocytes, such as alveolar macrophages and neutrophils (Gueirard *et al.*, 1998; Harvill *et al.*, 1999). The importance of CyaA in resisting host defense mechanisms was elegantly demonstrated using SCID/Beige mice, defective in the development of T and B cells and NK cell activity, and dependent on constitutive, non-adaptive defense mechanisms for protection against microbial pathogens. Wild type *B. bronchiseptica* killed SCID/Beige mice within about 50 days, while CyaA-deficient mutants did not even cause noticeable disease. Thus, CyaA overcomes the defense mechanisms that are retained in these animals. Furthermore, when mice were rendered neutropenic, they were killed by both wild type and *cyaA* mutant *B. bronchiseptica*, demonstrating that in the absence of neutrophils, *cyaA* is not required (Harvill *et al.*, 1999; Cotter and Miller, 2001). The definitive proof that CyaA specifically targets the myeloid

phagocytic cells came from identification of CyaA receptor, integrin CD11b/CD18, being specifically expressed by macrophages, neutrophils and dendritic cells (Guermonprez *et al.*, 2001).

Interaction of CyaA with phagocytes

Upon CD11b/CD18 recetor binding, the AC domain of CyaA is translocated to the cell cytosol where it is activated by host cell calmodulin and catalyzes the production of supraphysiologic levels of cAMP from ATP, exhibiting a tremendous catalytic power of $k_{cat} \sim 2000\ s^{-1}$. This appears to be about three orders of magnitude higher than the catalytic activity of the endogenous cellular adenylate cyclase enzymes (Ladant and Ullmann, 1999).

CyaA-mediated cytosolic cAMP elevation then causes phagocyte impotence (Confer and Eaton, 1982). In particular, cAMP signaling of CyaA was reported to impair chemotactic and oxidative burst capacities of phagocytes (Friedman *et al.*, 1987; Galgiani *et al.*, 1988; Pearson *et al.*, 1987) and to inhibit FcR-mediated phagocytosis by neutrophils (Weingart *et al.*, 2000; Weingart and Weiss, 2000). The toxin might also eventually induce macrophage apoptosis (Gueirard *et al.*, 1998; Khelef and Guiso, 1995; Khelef *et al.*, 1993) by a mechanism involving disruption of the membrane potential in mitochondria (Bachelet *et al.*, 2002). Pro-apoptotic activity of CyaA may be also potentiated by CyaA-induced downregulation of the expression of the heat shock protein (Hsp70), that increases the resistance to apoptosis in several cell types (Bachelet *et al.*, 2002; Njamkepo *et al.*, 2000). CyaA at higher concentrations was reported to cause phagocyte lysis (necrosis), primarily due to a massive ATP depletion (Basler *et al.*, 2006).

Immunomodulatory activity of CyaA

Besides subversion of bactericidal functions of immune cells, CyaA was also reported to have an immunomodulatory activity on DCs as well as on macrophages. CyaA of *B. pertussis* enhanced TLR-ligand-induced production of IL-6 and IL-10 cytokines, while inhibiting the TNF-α, IL-12 and CCL3 (MIP-1α) production by DCs and macrophages *in vitro* (Boyd *et al.*, 2005; Ross *et al.*, 2004). Further, CyaA up-regulated surface expression of the costimulatory molecule CD80 on immature DCs, but decreased expression of LPS-induced ICAM-1 (CD54) and CD40 molecules, which indicated that CyaA action selectively modulates DC activation and maturation (Ross *et al.*, 2004). Interestingly, CyaA of *B. pertussis* is an effective vaccine adjuvant *in vivo*, enhancing antibody response to coadministered antigens (Hormozi *et al.*, 1999; Macdonald-Fyall *et al.*, 2004), and was also

shown to promote Th2 and type 1 regulatory T (Tr1) cell responses to coadministered antigens (Ross *et al.*, 2004). In fact, DC-driven expansion of Treg cells might prevent infection-induced immunopathology and prolong pathogen persistence (Lutz and Schuler, 2002; McGuirk *et al.*, 2002; McGuirk and Mills, 2002; Mills and McGuirk, 2004).

Experiments with live *B. bronchiseptica* demonstrated that CyaA and effectors injected into cells by type III secretion system (TTSS) synergize in driving DCs into a semi-mature state, likely via modulation of mitogen-activated protein kinase (MAPK) pathways (Reissinger *et al.*, 2005; Skinner *et al.*, 2004). CyaA and TTSS are also required for the inhibition of antigen-driven $CD4^+$ T cell proliferation by *B. bronchiseptica*-infected murine bone marrow-derived macrophages, and the induced PGE_2 production was identified as the mediator of this inhibition (Siciliano *et al.*, 2006). *B. bronchiseptica*-infected macrophages further preferentially induce a Th17 profile of $CD4^+$ T cells, which partially depends on the presence of CyaA and TSSS (Siciliano *et al.*, 2006).

Interaction of CyaA with epithelial cells

B. pertussis is killed upon incidental invasion into epithelial cells (Gueirard *et al.*, 2005), and the bacteria are considered to remain extracellular when colonizing and adhering to the ciliated respiratory epithelium. CyaA action was suggested to represent an anti-invasive mechanism of *B. pertussis,* employed to avoid destruction within tracheal epithelial cells, as CyaA-deficient mutants of *B. pertussis* were found to be more invasive into human tracheal epithelial cells than the parental strain (Bassinet *et al.*, 2000). Besides that, CyaA activity may account for induction of IL-6 in tracheal epithelia colonized by *B. pertussis* (Bassinet *et al.*, 2004). The biological consequence of this CyaA-induced IL-6 production and the role of CyaA in interaction of *Bordetella* with airway epithelial cells and in shaping of the host immune response, however, await further investigation.

CyaA biogenesis

The expression of CyaA toxin from the *cya* locus is controlled by the Bvg two-component system, the regulator of *Bordetella* virulence genes (Scarlato *et al.*, 1991). Of the five *cya* genes in the locus, the *cyaABDE* genes are grouped within an operon, while the *cyaC*

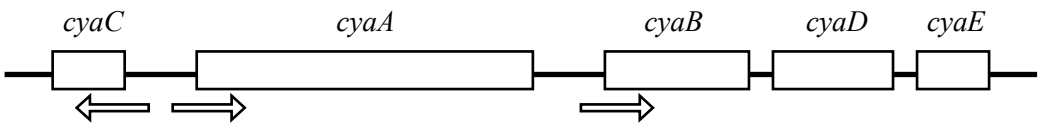

Fig. 7. Schematic representation of the *cya* locus. *cyaC* – acyltransferase; *cyaA* – adenylate cyclase toxin; *cyaB, cyaD, cyaE* - components of a type I secretion system; *cyaB* – inner membrane ATPase; *cyaD* – membrane fusion protein; *cyaE* – outer membrane channel component.

gene is transcribed in an opposite direction (Figure 7). The *cyaABDE* operon starts by the structural *cyaA* gene encoding proCyaA, followed by three genes encoding the components of a type I protein secretion system: (i) CyaB (the inner membrane ATPase, homolog of HlyB); (ii) CyaD (the membrane fusion protein, homolog of HlyD); and CyaE (the outer membrane channel component, homolog of TolC); (Glaser *et al.*, 1988; Laoide and Ullmann, 1990). The *cyaC* gene encodes a 22 kDa CyaC acyltransferase, which catalyzes the posttranslational palmitoylation of proCyaA and converts proCyaA into mature and biologically active CyaA toxin (Barry *et al.*, 1991).

Like other RTX toxins, CyaA is secreted by the bacterial type I secretion system. The mechanism of CyaA secretion is likely identical to that used for secretion of the RTX α-hemolysin (HlyA) of *E. coli* through the HlyAB/TolC apparatus (Figure 8), (Koronakis *et al.*, 2004). CyaB would operate as an ABC family ATPase that would recognize the C-terminal secretion signal of 74 residues of CyaA (Sebo and Ladant, 1993) and would drive the secretion of an unfolded CyaA polypeptide through a tightly packed trimeric CyaBDE conduit. The secretion of CyaA would occur directly from bacterial cytoplasm into the external medium, without the passage of CyaA through the periplasmic compartment (Masin *et al.*, 2006).

Some of the secreted CyaA molecules interact with the filamentous haemaglutinin (FHA) on the surface of *Bordetella* and remain surface-associated (Hewlett *et al.*, 1976;

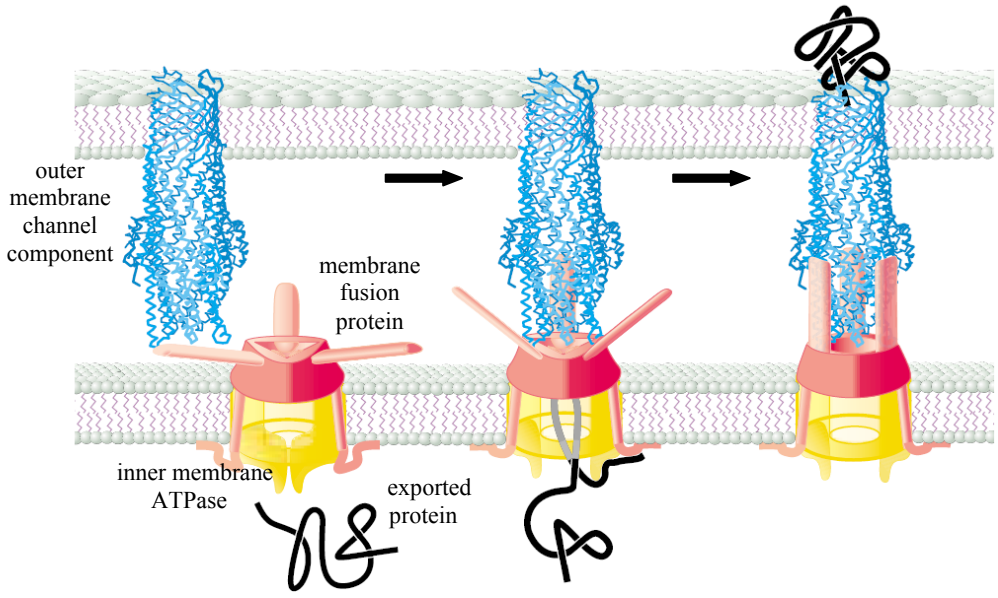

Fig. 8. Depiction of protein export through type I secretion system. Trimeric outer membrane channel component (blue) would reversibly interact with substrate-specific complex that contains a membrane fusion protein (red), an inner membrane ATPase (yellow) and the exported protein (black). The periplasmic tunnel would be contacted by the membrane fusion protein, possibly via the predicted coiled-coil structures, triggering the conformational change that opens the entrance to the exit duct. Following export, the components would revert to the resting state (Koronakis *et al.*, 2004).

Zaretzky *et al.*, 2002). They, however, appear to be aggregated and unable to act like a "contact weapon", as only the newly secreted CyaA molecules, not the bacteria-associated ones, were found capable of cell penetration and raising of intracellular cAMP levels (Gray *et al.*, 2004). Interestingly, this bacterial cell-associated CyaA still appears to be of potential benefit to the producing bacteria, as Edwards *et al.* (2005) showed that CyaA associated with cell surface of *B. bronchiseptica* contributes to the ability of the bacteria to adhere to ciliated epithelium (Edwards *et al.*, 2005).

Structure of CyaA molecule

The CyaA is a single polypeptide of 1,706 residues and consists of an amino-terminal AC domain of ~400 residues and a pore-forming hemolysin (Hly) moiety of ~1,300 residues (Figure 9), (Ladant and Ullmann, 1999). The Hly moiety harbors the following: (i) a hydrophobic pore-forming domain (residues 500 – 800) (Basler *et al.*, 2007; Benz *et al.*, 1994; Osickova *et al.*, 1999); (ii) an acylation domain (residues 800 – 1,000), where the posttranslational activation takes place (Hackett *et al.*, 1994; Hackett *et al.*, 1995); (iii) a RTX domain that harbors the characteristic calcium-binding repeats of consensus X-(L/I/F)-X-G-G-X-G-(N/D)-D sequence (Rhodes *et al.*, 2001; Rose *et al.*, 1995), and (iiii) a C-terminal type I secretion signal (Sebo and Ladant, 1993).

The AC domain is enzymatically active by itself (Glaser *et al.*, 1989), but the entire toxin is needed for AC delivery into cell cytosol (Bellalou *et al.*, 1990), where it is activated by binding of intracellular calmodulin and catalyzes ATP to cAMP conversion (Wolff *et al.*,

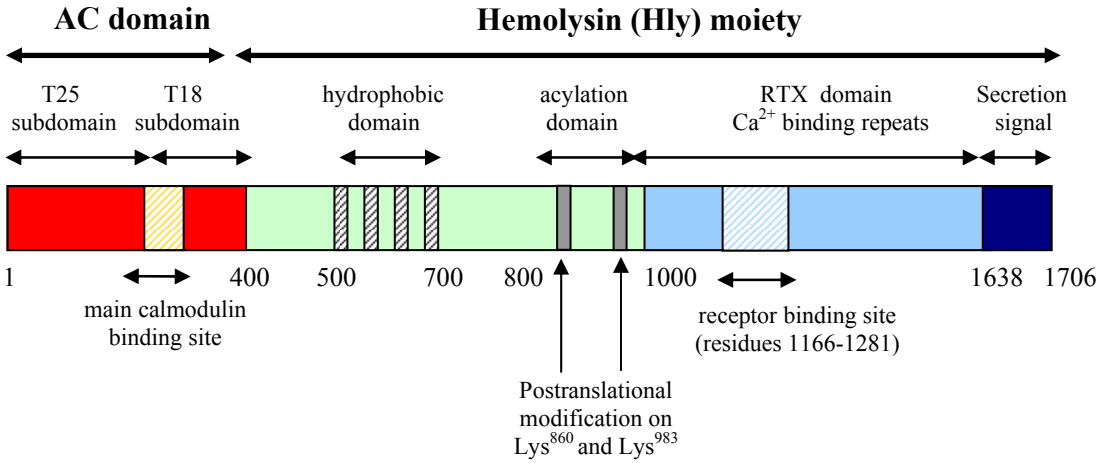

Fig. 9. Structural organization of the 1706 residue-long *B. pertussis* CyaA protein. The N-terminal catalytic domain of 400 residues is a cell-invasive and calmodulin-activated adenylate cyclase (AC) enzyme; T25 and T18 correspond to subdomains of AC. The last 1300 residues represent an RTX hemolysin moiety (Hly) of CyaA. This harbors (i) the hydrophobic pore-forming domain, (ii) the acylation domain, where post-translational activation of the protoxin is accomplished through CyaC-mediated covalent fatty-acylation of either of the ε-amino groups of Lys^{860} and Lys^{983}, (iii) the RTX domain with Ca^{2+} binding repeats, mediating the interaction with β_2 integrin receptor CD11b/CD18, and (iv) the C-terminal type I secretion signal (Vojtova *et al.*, 2006).

1980). In turn, the Hly moiety of CyaA is functionally independent and is itself capable to form small cation-selective membrane pores of a diameter between 0.6 to 0.8 nm (Benz *et al.*, 1994; Sakamoto *et al.*, 1992). These can permeabilize cellular membrane and provoke colloid-osmotic lysis of erythrocytes, which accounts for the hemolytic halo of *Bordetella* colonies on blood agar plates. Although the specific hemolytic activity of CyaA is weak, when compared with hemolysins of the RTX family (Bellalou *et al.*, 1990; Ehrmann *et al.*, 1991), it synergizes with the AC enzyme activity in maximizing the overall cytolytic potency of CyaA on monocytes (Basler *et al.*, 2006; Hewlett *et al.*, 2006).

Requirement for binding of Ca^{2+} ions in toxin activity of CyaA

CyaA activity strictly requires exposure of the protein to physiological (>0.3 mM) concentrations of free Ca^{2+} ions. The binding of Ca^{2+} ions to the CyaA molecule probably triggers an "active" conformation of the CyaA and allows for toxin binding and penetration of cellular membranes as well as for formation of cation-selective pores (Hanski and Farfel, 1985; Hewlett *et al.*, 1991; Rogel and Hanski, 1992). Moreover, Ca^{2+} ions are required also for CyaA binding to the $\alpha_M\beta_2$ integrin receptor (CD11b/CD18), (Guermonprez *et al.*, 2001).

The toxin harbors numerous (~35-40) low-affinity calcium-binding sites (Kd >0.3 mM) and a small number (~5) of high-affinity sites (Kd <1 nM). While the localization of the high affinity binding sites within the Hly moiety of CyaA remains unknown, the low-affinity Ca^{2+} binding sites were located within the last 700 C-terminal residues of CyaA (Rose *et al.*, 1995). By analogy to structure of the RTX moiety of the alkaline protease of *Pseudomonas* (Baumann *et al.*, 1993), the Ca^{2+} ions are expected to bind to CyaA within the turns connecting adjacent anti-parallel beta strands formed by the X-(L/I/F)-X-G-G-X-G-(N/D)-D nonapeptides of the RTX domain (Rhodes *et al.*, 2001). Calcium ions bind to CyaA in an extremely cooperative manner and the pore-forming activity of CyaA was found to be enhanced by ~50-fold upon a minimal (15%) increase of the free Ca^{2+} concentration from 0.6 to 0.8 mM (Knapp *et al.*, 2003; Masin *et al.*, 2006).

Postranslational-modification of CyaA

CyaA binding and penetration on cells lacking CD11b/CD18 receptor, as well as the productive binding to the CD11b/CD18 integrin receptor requires postranslational modification of CyaA within the acylation domain (El-Azami-El-Idrissi *et al.*, 2003; Rogel *et al.*, 1989). This is mediated by a co-expressed accessory protein, CyaC (Barry *et al.*, 1991; Sebo *et al.*, 1991), which catalyzes covalent palmitoylation of the ε-amino groups of internal

Lys^{860} and Lys^{983} residues of CyaA (Hackett *et al.*, 1994; Hackett *et al.*, 1995). Intriguingly, acylation of only Lys^{983} was found to be necessary and sufficient for toxin activity on the so far examined target cell types, lacking the CD11b/CD18 receptor (Basar *et al.*, 2001). On the other hand, high-affinity binding of CyaA to CD11b/CD18 is conferred by acylation of either Lys^{860}, or of Lys^{983}, where the CyaA acylated solely on Lys^{860} still exhibits a fairly high cytotoxic activity towards murine $CD11b^+$ monocytes (Masin *et al.*, 2005). This suggests that acylation of the two lysine residues is partially redundant in function and it remains unclear whether acylation of both of the lysines is required for toxin activity on some as yet unknown targets (Masin *et al.*, 2006). The character of acyl residues attached to CyaA, however, modulates specific hemolytic activity of the toxin. The recombinant *r-Ec*-CyaA which is modified in *E. coli* mainly by the unsaturated palmitoleil (*cis* Δ9 C16:1) fatty-acyl groups, exhibits reduced hemolytic activity, as compared to the *Bp*-CyaA, produced by *B. pertussis* and acylated by C16:0 palmitoyl chains. Both of CyaA proteins, however, still posses an equal capacity to penetrate cellular membranes of target cells (Benz *et al.*, 1994; Hackett *et al.*, 1995; Havlicek *et al.*, 2001).

Membrane interaction and penetration of CyaA

The current model of CyaA interaction with CD11b/CD18 target cell membrane is summarized in Figure 10. Acylated and calcium-loaded CyaA toxin in solution interacts with the CD11b/CD18 receptor on the membrane of target cells, which facilitates the insertion of the toxin into the lipid bilayer (Guermonprez *et al.*, 2001; Masin *et al.*, 2005). The main segment of CyaA required for binding to the CD11b/CD18 receptor was located between residues 1166-1281 of the RTX domain of CyaA (El-Azami-El-Idrissi *et al.*, 2003). On the site of integrin receptor, N-linked glycosylation of CD11b/CD18 appears to be crucial for CyaA binding (Morova *et al.*, 2008) and likely mediates the first CyaA-receptor contact, while a non-glycosylated segment of the CD11b subunit appears to determine the specificity of CyaA-receptor binding (Osicka *et al.,* unpublished).

After CD11b/CD18 binding and CyaA membrane insertion, two alternative events would occur (Osickova *et al.*, 1999): (a) penetration of AC domain to cell cytosol, or (b) formation of a cation-selective pores after CyaA oligomerization (Vojtova-Vodolanova *et al.*, 2009). These events are more-or-less mutually exclusive and operating in parallel, as the penetration of the AC domain into cells can be dissociated from formation of the cation-selective CyaA pores by the extent and nature of CyaA acylation (Hackett *et al.*, 1995) and by point substitutions in the predicted membrane segments of CyaA (Basler *et al.*, 2007;

Osickova *et al.*, 1999).

Importantly, the AC domain which translocates into cytosol of $CD11b^+$ cells, participates in formation of a novel membrane path for influx of Ca^{2+} ions, yielding elevation of cytosolic calcium concentration. Cytosolic elevation of Ca^{2+} ions does not depend on cell permeabilization by the CyaA cation-selective pores, or on cAMP production (Fiser *et al.*, 2007) and induces mobilization of the CyaA-receptor complex into lipid rafts via activation of calpain and cleavage of the talin (Figure 11), (Bumba *et al.,* submitted). Within the specific lipid environment of the membrane rafts, the penetration of the positively charged AC domain into cell cytosol, along the gradient of negative membrane potential would be completed (Otero *et al.*, 1995; Bumba *et al.,* submitted). The hydrophilic path to facilitate the AC domain penetration through this hydrophobic lipid environment would likely be formed by the transmembrane α-helices localized in the hydrophobic domain of CyaA and harboring two pairs of negatively charged glutamic acid residues (Glu^{509}/Glu^{516} and Glu^{570}/Glu^{581}) which were found to control AC domain translocation (Basler *et al.*, 2007; Osickova *et al.*, 1999).

Following penetration into target cells, the AC domain binds intracellular calmodulin at a 1:1 stoichiometry, which increases its enzymatic activity by >1000-fold, reaching the extremely high catalytic power of k_{cat} ~2000 s^{-1} (Ladant and Ullmann, 1999). Two functional subdomains could be defined within the AC domain, namely, the T25 subdomain (residues

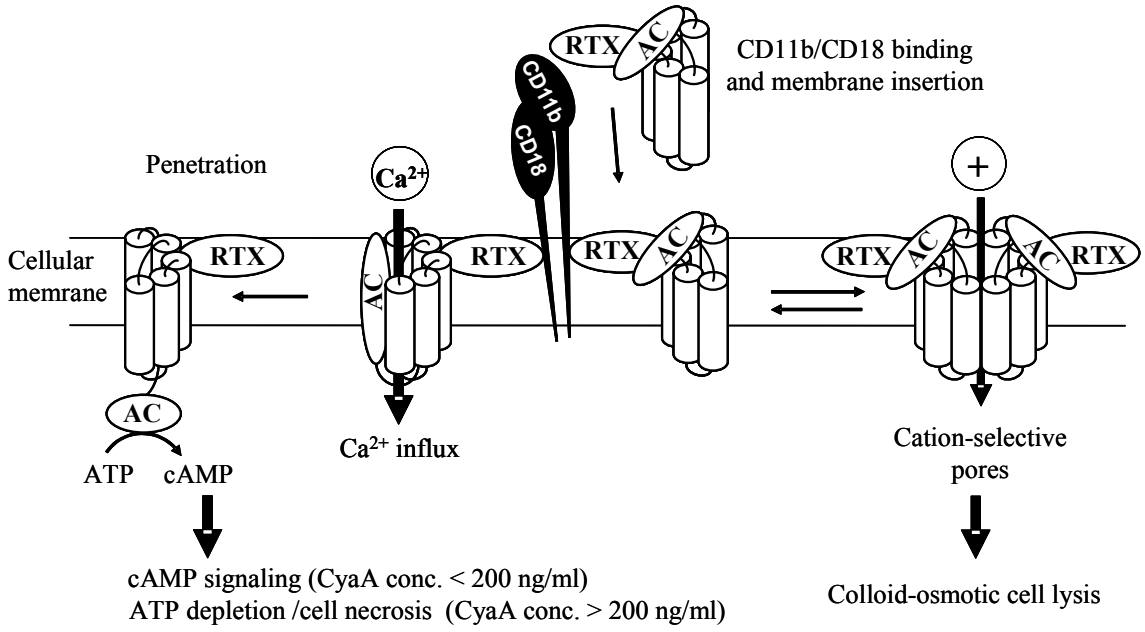

Fig. 10. Model of CyaA interaction with CD11b/CD18 target cell membrane. Acylated and calcium-loaded CyaA toxin interacts with CD11b/CD18 receptor. Upon membrane insertion, the CyaA toxin would either oligomerize and form the cation-selective pores, or mediate Ca^{2+} influx into the cells and translocate the AC domain into cell cytosol (Basler *et al*., 2007; Fiser *et al*., 2007; Osickova *et al*., 1999; Vojtova-Vodolanova *et al*., 2009). Once the AC domain is delivered into cell cytosol, it binds calmodulin and catalyzes conversion of ATP to cAMP, a key signaling molecule. At higher toxin concentration, massive ATP depletion occurs, yielding cell necrosis (Basler *et al*., 2006).

1-224) that harbors the AC catalytic site, and the T18 subdomain (residues 225-399) that posses the main calmodulin-binding site (Glaser *et al.*, 1989; Ladant, 1988; Ladant *et al.*, 1989). The crystal structure of the AC domain of CyaA in complex with the C-terminal fragment of calmodulin was recently solved by Guo *et al.* (2005). Four discrete regions of the AC domain bind to calcium-loaded calmodulin with a large contact surface, where a tryptophan residue 242 of the AC domain plays a crucial role and makes extensive contacts with the calcium-induced, hydrophobic pocket of calmodulin (Guo *et al.*, 2005).

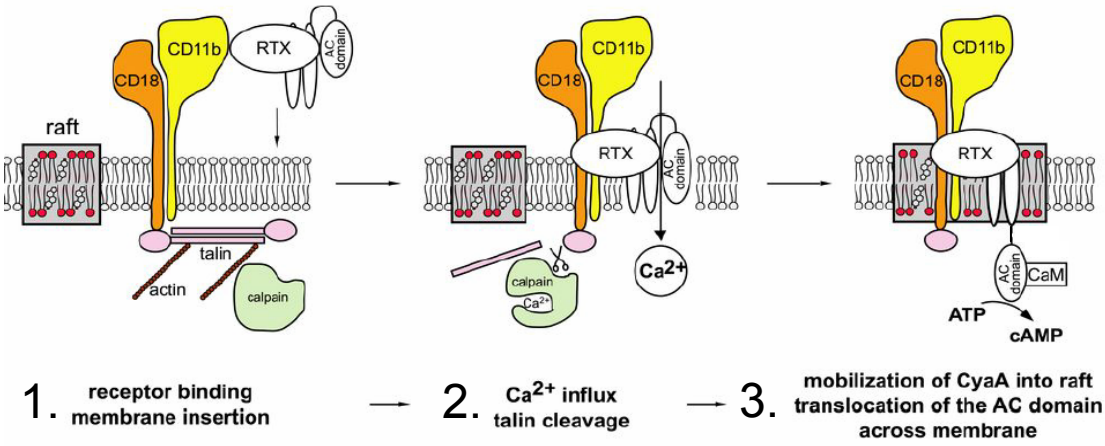

Fig. 11. Model of CyaA penetration into cells. In the first step, CyaA binds the CD11b/CD18 integrin receptor dispersed in the bulk of the membrane phase outside of lipid rafts, having the cytoplasmic tail of the CD18 subunit tethered to actin cytoskeleton *via* the linker protein talin. Upon receptor engagement, the AC domain partially inserts into cell membrane and participates in formation of a Ca^{2+}-conducting path across cell membrane. Influx of external Ca^{2+} into cells induces activation of the Ca^{2+}- dependent protease calpain, yielding talin cleavage and liberation of the CyaA-CD11b/CD18 complex from binding to actin cytoskeleton. Consequently, the complex is recruited into cholesterol-enriched membrane microdomains (rafts), where the specific lipid composition allows completion of AC domain penetration across the target cell membrane (Bumba *et al.*, submitted).

Utilization of CyaA as a marker enzyme tool

The AC-fusion reporter system for tracing protein translocation into eukaryotic cell cytosol was elegantly derived from the observations that in the absence of calmodulin the AC domain exhibits only a residual enzyme activity and that Gram-negative bacteria generally do not express calmodulin homologues (Sory and Cornelis, 1994). When the AC domain (as a reporter protein) is fused to an effector protein secreted through the type III secretion system (TTSS), such as a *Yersinia* Yop protein, the fusion exhibits only very low AC enzyme activity in the bacterial cell and/or culture supernatants. Once it is injected through the TTSS into a eukaryotic host cell, however, the AC enzyme is activated by >1000-fold by host calmodulin and catalyzes rapid conversion of ATP to cAMP. This can be easily assayed as intracellular

cAMP accumulation that is proportional to the amount of injected AC fusion protein. The AC-fusion reporter system has now become a standard technique in the TTSS field and is used for demonstration of contact-dependent direct translocation into animal and plant host cells of TTSS effectors of a number of Gram-negative species (Masin *et al.*, 2006).

Moreover, the bacterial two-hybrid system for detection of protein-protein interactions was developed on the observation that the residual activity of the AC domain in the absence of calmodulin requires the physical interaction of the T25 and T18 subdomains of the AC domain (Figure 12), (Karimova *et al.*, 1998b). When the T25 and T18 subdomains are individually fused to peptides or proteins that are able to bind each other, the interaction of the chimeric polypeptides yields a functional complementation of the T25 and T18 fragments and the restoration of a residual capacity of the enzyme to catalyze ATP conversion to cAMP. This, in turn, can be monitored by using *E. coli*, lacking the endogenous AC activity, as indicator strain. The cAMP which is produced upon functional interactions between expressed proteins, activates transcription of genes involved in lactose and maltose catabolism and allows for positive selection in minimal media, with maltose as unique carbon source, or for detection on indicator agar plates (Dautin *et al.*, 2002; Masin *et al.*, 2006).

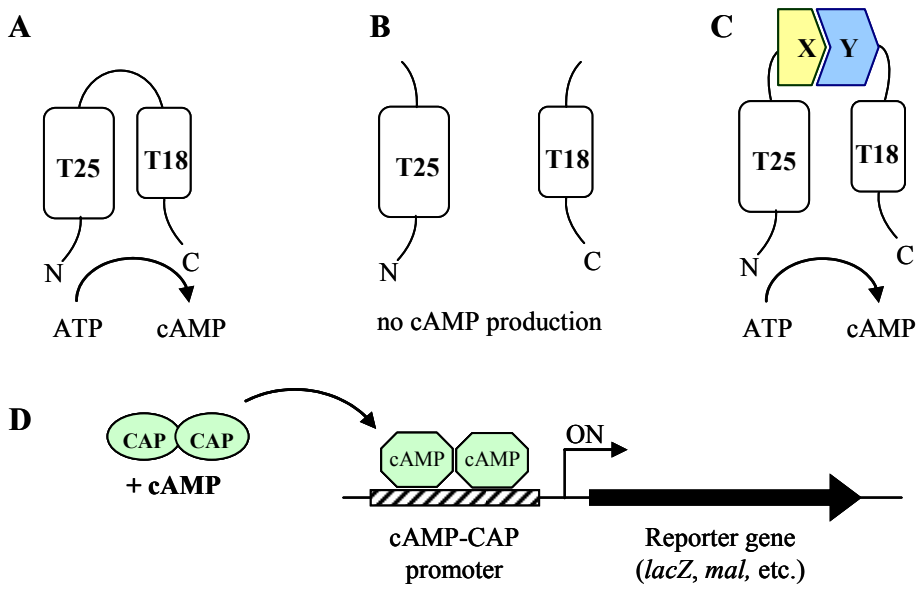

Fig. 12. CyaA as a reporter of protein–protein interactions in an *E. coli* cya⁻ strain. A) T25 (1-224 aa) and T18 (225-399 aa) subdomains of the AC domain expressed as single polypeptide; **B)** T25 and T18 co-expressed as separate entities; **C)** Interaction between polypeptides X and Y, fused to T25 and T18, respectively, results in functional complementation between the T25 and T18 and restoration of cAMP synthesis; **D)** Cyclic AMP binds to the catabolite activator protein (CAP), and the resulting cAMP–CAP complex can turn on the transcription of catabolic operons, reported genes (Ladant and Ullmann, 1999).

Antigen delivery by *Bordetella* adenylate cyclase

Genetically detoxified CyaA toxoids (CyaA/AC$^-$), unable to form cAMP, were successfully used as vectors for delivery of various inserted immunodominant T-cell epitopes into antigen presenting cells, yielding induction of T cell responses both *in vitro* and *in vivo* (Figure 13); (El Azami El Idrissi *et al.*, 2002; Simsova *et al.*, 2004). Indeed, phase I/II human clinical trials aiming at exploring this application of CyaA are currently in preparation.

The utilization of *Bordetella* adenylate cyclase toxin for antigen delivery relies on following CyaA properties: (i) CyaA is specifically targeted to myeloid phagocytic cells capable of antigen presentation, as these cells express the receptor recognized by CyaA, integrin CD11b/CD18 (Guermonprez *et al.*, 2001); (ii) CyaA with largely eliminated cytotoxicity due to mutations that ablate the AC enzyme activity, is still capable of CD11b/CD18 binding and AC domain delivery into cell cytosol (Guermonprez *et al.*, 1999);

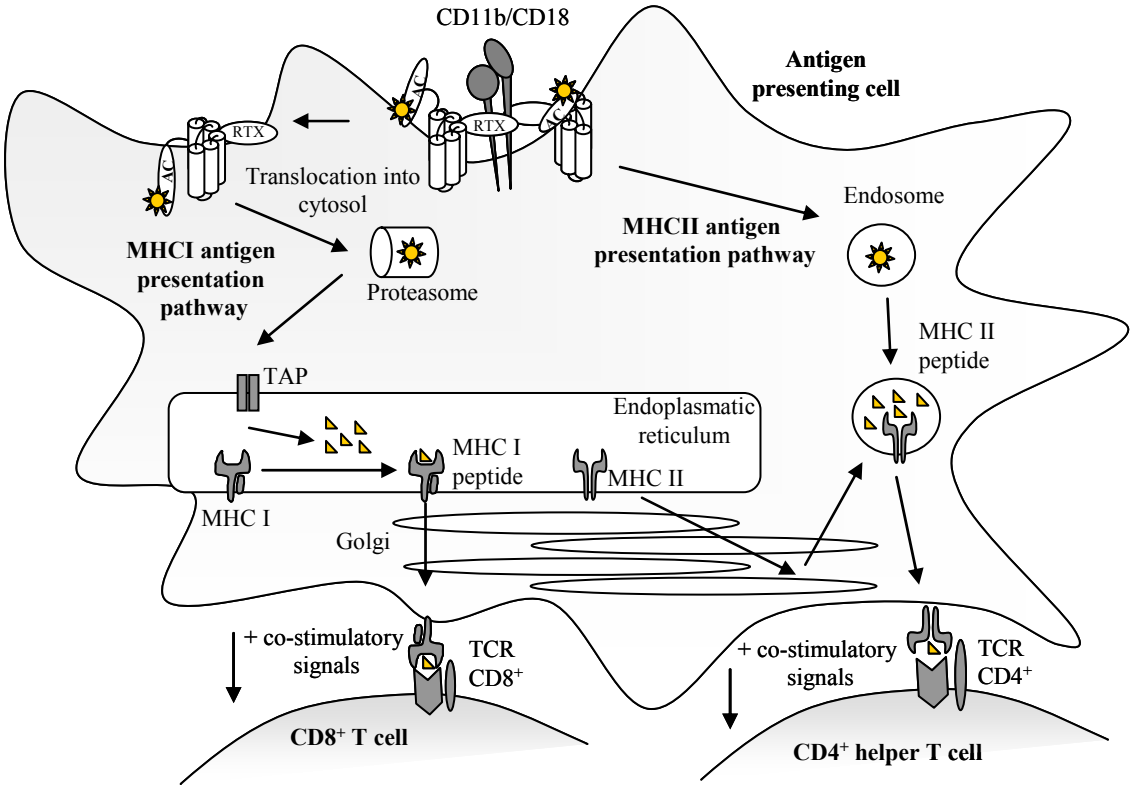

Fig. 13. CyaA-mediated delivery of antigens for processing and presentation on MHC class I and class II molecules. Upon CD11b/CD18 binding and insertion into the cell membrane, part of CyaA molecules translocates the AC domain into the cell cytosol, whereas the other part forms oligomeric membrane channels with the AC domain stuck at the external face of the cell membrane. Once the AC domain bearing the inserted immunodominant T-cell epitopes penetrates into the cytosol, it is processed by proteasome, and the resulting peptides are transported by TAP (transporter-associated with antigen procesing) into the endoplasmic reticulum, where they bind to the newly synthesized MHC class I molecules (Guermonprez *et al*., 1999). The resulting MHC class I-peptide complexes are transported to the cell surface and presented to CD8$^+$ T cells, thereby alowing induction of CD8$^+$ T cell responses. Receptor-mediated endocytosis of membrane-associated CyaA may also occur and CyaA molecules can be processed to antigenic peptides that bind to MHC class II molecules. These MHC class II-peptide complexes are presented to CD4$^+$ T cells, thereby alowing also induction of CD4$^+$ T cell responses (Loucka *et al*., 2002; Schlecht *et al*., 2004); adapted from (Simsova *et al*., 2004).

and (iii) AC domain of CyaA can accommodate foreign antigenic polypeptides without affecting the receptor-binding and cell-penetrating capacities of the toxoid (El-Azami-El-Idrissi *et al.*, 2003; Gmira *et al.*, 2001; Ladant *et al.*, 1992; Osicka *et al.*, 2000). This enables delivery of inserted immunodominant $CD8^+$ cytotoxic T lymphocyte (CTL) epitopes for cytosolic processing and presentation in association with MHC class I molecules, thereby allowing induction of strong and epitope-specific CTL responses (Dadaglio *et al.*, 2003; Fayolle *et al.*, 1996; Guermonprez *et al.*, 1999). Immunization of mice with CyaA/AC^- toxoids bearing appropriate T-cell epitopes, indeed, provides efficient prophylactic and therapeutic antitumor immunity (Fayolle *et al.*, 1999; Mackova *et al.*, 2006; Preville *et al.*, 2005). CyaA/AC^- toxoids were also effective in prophylactic vaccination against a lethal challenge of mice by the LCMV virus (Saron *et al.*, 1997).

In parallel, however, CyaA was also shown to enter CD11b/CD18-mediated endocytosis and to deliver immunodominant $CD4^+$ epitopes for presentation in association with MHC class II molecules. This can yield to induction of Th1-biased $CD4^+$ T cell responses (Dadaglio *et al.*, 2000; Loucka *et al.*, 2002; Schlecht *et al.*, 2004). Moreover, improvement of the sensitivity of immunodiagnostic test due to targeting of $CD11b^+$ cells by CyaA toxoids bearing appropriate epitopes has been recently reported (Vordermeier *et al.*, 2004). Thus, CyaA toxoids have also the potential to be developed into sensitive diagnostic reagents for improving the current immunodiagnostic tests.

AIMS

Part I.: Molecular mechanism of action of the adenylate cyclase toxin

Adenylate cyclase toxin (CyaA), a member of the family of RTX (Repeat in ToXin) proteins, is a key virulence factor of the whooping cough agent *Bordetella pertussis*. Upon binding the β_2 integrin CD11b/CD18 receptor, the toxin permeabilizes cellular membranes by forming small cation-selective pores, and delivers its adenylate cyclase (AC) domain directly across cytoplasmic membrane into cell cytosol.

The mechanism of penetration of the AC domain directly into the cytosol of target cells across cytoplasmic membrane is poorly understood. The cell-penetrating activity of CyaA was shown to depend on plasma membrane potential and on an intact, acylated and calcium-loaded Hly moiety. The predicted transmembrane amphipathic α-helices between residues 502-522 and 565-591 of the hydrophobic domain of CyaA, harboring pairs of negatively charged glutamate residues Glu^{509}/Glu^{516} and Glu^{570}/Glu^{581}, were indeed recently shown to be directly involved in AC domain penetration across cell membrane. It remained, however, unclear whether there were any segments within the AC domain that cooperate with the Hly moiety of CyaA in the process of AC domain translocation across cytoplasmic membrane.

AC domain upon its penetration to cell cytosol is activated by binding of eukaryotic calmodulin and catalyzes unregulated conversion of cellular ATP to cAMP, a key signaling molecule. Rapid elevation of intracellular cAMP concentration by CyaA in $CD11b^+$ phagocytes was shown to yield suppression of phagocyte bactericidal functions, such as chemotaxis, FcR-mediated phagocytosis, or superoxide production and eventually to provoke apoptosis. This capacity of CyaA appears to account for the key role of CyaA in virulence of *Bordetella* species in mammals, enabling the bacteria to escape destruction by the first-line defense of innate immune system. However, although suppressive effects of CyaA action on functions of myeloid phagocytes were repeatedly reported, little attention was paid to the underlying mechanisms of toxin-mediated cAMP signaling and to the corresponding downstream effectors, which remained to be identified.

It was suggested that cAMP signaling of CyaA may play an even more versatile role in fooling the host defense by promoting incomplete or aberrant maturation of dendritic cells (DCs), thereby shaping the induction of adaptive T cell immune responses. In mamalian cells, cAMP signaling is mediated through activation of protein kinase A (PKA), the guanine exchange proteins directly activated by cAMP (Epac-1 and Epac-2) and the cAMP-activated channels, respectively. While the role of Epac1 signaling pathway in the control of the macrophage functions is emerging, the role of Epac1 signaling pathway in DC function was unclear.

To clarify these issues, we pursued the following specific aims**:**

1. **Mutagenize the AC domain and determine the penetration capacities of mutant toxin variants in order to identify sequences of the AC domain contributing to its delivery across cell plasma membrane**

2. **Characterize the toxin-mediated subversion of bactericidal functions of phagocytes and identify the underlying signaling mechanisms**

3. **Evaluate the toxin capacity to impair initiation of $CD4^+$ and $CD8^+$ T cell adaptive responses through modulation of dendritic cells and determine the role of Epac signaling pathway in control of dendritic cell functions**

Part II.: Use of adenylate cyclase toxoid for antigen delivery

The adenylate cyclase toxoid (CyaA/AC$^-$), which has the cytotoxic activity of CyaA largely eliminated by mutation that ablate the catalytic AC activity, is still capable of delivering its N-terminal catalytic domain into the cytosol of CD11b$^+$ professional antigen-presenting cells (APCs), such as dendritic cells (DCs). This allows the use of CyaA/AC$^-$ for delivery of inserted cargo CD8$^+$ T-cell epitopes into cytosol of APCs, thus enabling cytosolic processing and subsequent cell surface presentation of the epitope cargo on MHC class I molecules and induction of strong and epitope-specific cytotoxic CD8$^+$ T lymphocyte (CTL) responses. Immunization with CyaA/AC$^-$ bearing appropriate CD8$^+$ T-cell epitopes was, indeed, found to induce specific CTL responses against different model antigens, as well as to confer a full prophylactic protection against lethal lymphocytic choriomeningitis virus challenge and an efficient therapeutic antitumor immunity in mice, thus demonstrating the versatility of this tool as an antigen delivery system.

However, the adenylate cyclase toxoid was not, so far, used for induction of an anti-parasitic immune response. Protozoan parasites of the genus *Plasmodium* cause malaria, an infectious disease that represents a major public health problem in developing world. Each year, there are approximately 350–500 million cases of malaria, killing between one and three million people. A sterile protection against malaria can be achieved by the induction of IFNγ-producing CD8$^+$ T cells that target infected hepatocytes presenting epitopes of the circumsporozoite protein (CSP).

Therefore, we followed the specific aim:

To prepare and purify the recombinant CyaA toxoids bearing CD8$^+$ T-cell epitope of circumsporozoite protein (CSP) of *Plasmodium berghei.* These will be used to induce a specific CD8$^+$ T cell response in experimental mice malaria model, aiming to confer protection against challenge with *P. berghei* sporozoites.

PUBLICATIONS

List of publications

Part I.: Molecular mechanisms of action of the adenylate cyclase toxin

1) Vojtova J., **Kamanova J.** and Sebo P.: *Bordetella* adenylate cyclase toxin: a swift saboteur of host defense. Curr Opin Microbiol. 2006 Feb;9(1):69-75; $IF_{2008} = 7.483$.

2) Holubova J., **Kamanova J.**, Masin J. and Sebo P.: Adenylate cyclase domain is entrained across target cell membrane by the RTX moiety of *Bordetella* CyaA. *Manuscript in preparation for Infect Immun.*

3) **Kamanova J.,** Kofronova O., Masin J., Genth H., Vojtova J., Linhartova I., Benada O., Just I. and Sebo P.: Adenylate Cyclase Toxin Subverts Phagocyte Function by RhoA Inhibition and Unproductive Ruffling. J Immunol. 2008 Oct 15;181(8):5587-97; $IF_{2008} = 6.000$.

4) Adkins I., **Kamanova J.**, Tomala J., Janova H., Masin J., Kovar M. and Sebo P.: *Bordetella* adenylate cyclase toxin decreases $CD4^+$ and $CD8^+$ T cell expansion via modulation of dendritic cells. *Manuscript in preparation for Infect Immun.*

Part II.: Use of adenylate cyclase toxoid for antigen delivery

5) Tartz S., **Kamanova J.**, Simsova M., Sebo P., Bolte S., Heussler V., Fleischer B. and Jacobs T.: Immunization with a Circumsporozoite Epitope Fused to *Bordetella pertussis* Adenylate Cyclase in Conjunction with Cytotoxic T-Lymphocyte-Associated Antigen Blockade Confers Protection against *Plasmodium berghei* Liver-Stage Malaria. Infect Immun. 2006 Apr;74(4):2277-85; $IF_{2008} = 3.987$.

6) Tartz S., Rüssmann H., **Kamanova J.**, Sebo P., Sturm A., Heussler V., Fleischer B. and Jacobs T.: 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 Nov 5;26(47):5935-43; $IF_{2008} = 3.298$.

DISCUSSION

Part I.: Molecular mechanism of action of the adenylate cyclase toxin.

The adenylate cyclase toxin (CyaA) of *Bordetella pertussis* is composed of a single polypeptide of 1706 amino acid residues that accounts for all of the toxin features: (i) binding of the integrin CD11b/CD18 receptor and membrane insertion, (ii) formation of cation-selective pores within plasma membrane, and (iii) penetration of the adenylate cyclase (AC) domain (~400 residues) into cell cytosol. There, the AC domain binds intracellular calmodulin and catalyzes conversion of ATP to cAMP. The elevated cAMP levels then impair bactericidal functions of phagocytes, including chemotaxis, FcR-mediated phagocytosis and oxidative burst, and modulate maturation of DCs, thereby likely subverting also the adaptive immune responses of the host (Ladant and Ullmann, 1999; Vojtova *et al.*, 2006).

In the first part of this PhD. thesis, the molecular mechanism of action of the CyaA toxin was analyzed. The Publication 1 (2006) reviews the understanding of the interaction of CyaA with target cells and highlights the most important findings in the past five years. In the remaining publications, the following CyaA actions were unraveled, (i) delivery of the AC domain into cell cytosol (Publication 2), (ii) subversion of phagocyte functions by CyaA (Publication 3), and (iii) the ability of the toxin to impair T cell priming via modulation of DCs (Publication 4).

Penetration of AC domain of CyaA across cell plasma membrane – Publication 2

In contrast to majority of other bacterial toxins, CyaA reaches cell cytosol directly from plasma membrane, without the need for endocytosis (Ladant and Ullmann, 1999). Elevation of cytosolic cAMP levels occurs immediately upon cell exposure to CyaA toxin (Basler *et al.*, 2006; Gordon *et al.*, 1989), and the toxin was repeatedly shown to penetrate even into cells lacking endocytosis, such as sheep erythrocytes (Osicka *et al.*, 2000; Rogel and Hanski, 1992). Moreover, the CD18-dependent trafficking of CyaA, but not the accumulation of intracellular cAMP or the AC domain-mediated delivery of foreign MHC class I restricted T cell epitopes into cell cytosol is blocked upon inhibition of cell endocytosis (Gordon *et al.*, 1988; Guermonprez *et al.*, 1999; Schlecht *et al.*, 2004).

While it is fairly well established that CyaA penetrates directly across the cell plasma membrane, the mechanism of AC domain penetration into cell cytosol remains poorly understood. The cell penetrating activity of CyaA requires structural integrity of the acylated and calcium-loaded Hly moiety of the toxin (Bellalou *et al.*, 1990; Rogel and Hanski, 1992; Rogel *et al.*, 1989), depends on the surrounding lipid environment (Bumba *et al.*, submitted), and is presumably driven by the membrane potential (Otero *et al.*, 1995). Until recently, it was also unclear whether there were any segments within the AC domain that would actively cooperate with the Hly moiety of CyaA to mediate the AC domain delivery.

We deciphered here the contribution of the AC domain to its cytosolic penetration by analyzing 18 different deletions within the AC domain for toxoid delivering properties to MHC class I presentation pathway. This approach exploits the fact that efficient delivery into MHC class I presentation pathway and subsequent stimulation of $CD8^+$ T cell hybridoma by CyaA toxoids requires cytosolic penetration of the MHC class I restricted antigenic epitopes (Guermonprez *et al.*, 1999; Karimova *et al.*, 1998a).

We have shown that deletions of up to 371 amino acid residues in the AC domain, exhibit only minor or undetectable effects on CyaA-mediated cytosolic delivery of MHC class I restricted T cell epitope of ovalbumin and stimulation of $CD8^+$ T cell hybridoma. Importantly, the robustness and specificity of our assay was demonstrated by showing that the CyaA-E570K+E581P toxoid, which still binds CD11b/CD18 integrin receptor but lacks the cell invasive activity, fails to promote any detectable stimulation of the hybridoma. AC domain would, thus, act during the membrane penetration solely as a passive passenger, a cargo. The function of the AC domain in the wild type toxin would be exclusively to generate high levels of cytosolic cAMP, due to extreme catalytic power of $k_{cat} \sim 2000\ s^{-1}$, leading to subversion of host defenses (Vojtova *et al.*, 2006).

Indeed, several other bacterial toxins elicit their action by alteration of intracellular concentrations of cAMP, such as anthrax toxin of *Bacillus anthracis*, cholera toxin of *Vibrio cholerae*, pertussis toxin of *Bordella pertussis* and *E. coli* heat-labile enterotoxin. The anthrax toxin is also a calmodulin-activated adenylate cyclase which upon penetration to cell cytosol generates cAMP from endogenous ATP. The cholera toxin, pertussis toxin and *E. coli* heat-labile enterotoxin act within the cell by means of ADP-ribosylation of the GTP-binding proteins that regulate the host adenylate cyclases, and thereby increase cytosolic cAMP levels (Ahuja *et al.*, 2004). In contrast to *Bordetella* CyaA, these toxins are, however, multi-component A-B type toxins whose penetration require the presence of separate polypeptides and occur upon receptor-mediated endocytosis (Falnes and Sandvig, 2000).

Interestingly, concerning the use of CyaA toxoids for antigen delivery, the AC domain polypetide seems to be dispensable. This suggests the possibility of construction of CyaA toxoids carrying antigens fused directly to the Hly moiety of CyaA molecule. The Hly moiety, indeed, accounts for specific targeting to $CD11c^{+}$ $CD8\alpha^{-}$ $CD11b^{high}$ dendritic cells *in vivo* (El-Azami-El-Idrissi *et al.*, 2003; Guermonprez *et al.*, 2002), and mediates penetration of epitope cargo into cell cytosol of dendritic cells, as shown here. We are currently preparing CyaA toxoids to corroborate our hypothesis, and employ these toxoids in *in vivo* immunization schemes.

cAMP signaling of CyaA subverts phagocyte function – Publication 3

Within this study, we have confirmed that CyaA exhibits immunosuppressive effects on bactericidal functions of macrophages. For the first time, we have shown here that cAMP signaling upon exposure of macrophages to physiologically relevant concentrations of the CyaA toxin causes a rapid and complete inhibition of CR3-mediated phagocytosis, which may be of crucial importance in the early stages of bacterial colonization of naive (unvaccinated) infants that lack specific Abs to *B. pertussis*. We also demonstrated that this subversive action of CyaA was due to transient inactivation of RhoA, the signaling of which is shut down as a consequence of cAMP signaling.

In addition, we observed that the CyaA/cAMP-induced drop of RhoA activity yielded dephosphorylation of the actin filament severing protein cofilin and massive actin cytoskeleton rearrangements, which were paralleled by rapidly manifested macrophage ruffling. Control of actin cytoskeleton reorganization is, indeed, essential for a variety of fundamental cellular processes, such as cytokinesis, migration and cell adhesion, and intracellular trafficking processes, including phagocytosis and assembly of the NADPH oxidase complex, involved in oxidative burst (Anand *et al.*, 2008; Davis *et al.*, 2007; Chen *et al.*, 2007; Zhang *et al.*, 2009). Also, for example, actin cytoskeleton of antigen-presenting cells needs to be rapidly mobilized in response to signals of the innate immune system to enhance antigen capture and presentation (West *et al.*, 2004). Hence, the subversion of bactericidal activities of macrophages by CyaA toxin may well rely on disruption of actin cytoskeleton homeostasis and induction of membrane ruffling, through inhibition of RhoA signaling.

Intriguingly, Rac1 activity was also found here to be required for induction of membrane ruffling by CyaA. This could be explained in the light of the apparently

antagonistic relationship between Rac and RhoA, with the two proteins suppressing each other's activities and phenotypes (Burridge and Doughman, 2006; Caron, 2003). The membrane ruffling in monocyte/macrophage cells is mediated by the activation of signaling of Rac-like protein subfamily (Allen *et al.*, 1997; Burridge and Wennerberg, 2004), however activation of RhoA and its downstream effector, ROK may supress this membrane protrusive activity (Ohta *et al.*, 2006; Worthylake and Burridge, 2003; Xu *et al.*, 2003). Indeed, inhibition of RhoA/ROK signaling pathway enhances the membrane protrusive activity of primary monocytes and results in competing membrane lamellae (Worthylake and Burridge, 2003). Thus, the observed cAMP signaling-mediated inactivation of RhoA would be well compatible with induction of ruffling in macrophages.

Targeting the RhoA signaling pathway by CyaA in macrophages further goes well with previous reports showing that cAMP negatively regulates multiple components of the RhoA signaling pathway. RhoA can, indeed, be phosphorylated by cAMP-activated PKA at the Ser188 residue, near its C-terminus and this appears to inhibit RhoA function, (i) by impairing RhoA interaction with its effector ROKα (Dong *et al.*, 1998; Nusser *et al.*, 2006), and/or (ii) by enhancing RhoA interaction with the Rho guanine nucleotide dissociation inhibitor (RhoGDI), respectively (Ellerbroek *et al.*, 2003; Lang *et al.*, 1996; Tamma *et al.*, 2003). Recent studies indicate that PKA can also regulate the activity of upstream activators of RhoA, such as Gα13 (Manganello *et al.*, 2003), and/or inhibit the RhoA-GEF activity of the AKAP-Lbc complex by phosphorylation and recruitment of the 14-3-3 protein (Diviani *et al.*, 2004). Besides that, also activation of the cAMP/Epac1/Rap1 signaling pathway may contribute to RhoA inhibition, as it has been demonstrated that RhoA-GAP activity of ARAP3 is activated upon binding of Rap (Krugmann *et al.*, 2004).

Strikingly, membrane ruffling is intimately linked to the formation of macropinosomes, originating primarily as actin-rich ruffles that close to form intracellular vesicles. For example, M-CSF (macrophage colony stimulating factor) or PMA stimulate in macrophages both, membrane ruffling as well as macropinocytosis (Racoosin and Swanson, 1989; Swanson, 1989). Moreover, various pathogenic bacteria such as *Salmonella typhimurium* and *Shigella* spp. inject toxins into cells to trigger membrane ruffling and ensure their own uptake during cell invasion (Rottner *et al.*, 2005; Steele-Mortimer *et al.*, 2000). The opposite, however, appears to be the result of action of *Bordetella* CyaA-triggered membrane ruffling, and it will be of interest to elucidate the exact mechanism of how CyaA action leads to inhibition of macropinocytosis. While ruffling is a prerequisite for macropinosome formation, indeed, additional activities may be required to transform a ruffle into a closed

intracellular vesicle. For example, Araki *et al.* (1996) showed that PI 3-kinase activity is necessary for the completion of actin-dependent macropinocytosis (Araki *et al.*, 1996). In the light of the results reported here, it is hence plausible to hypothesize that cAMP/CyaA signaling may target also the signaling pathway accounting for closure of macropinosomes and/or phagosomes and their transformation into intracellular vesicles, which would go well with previous observations that CyaA is an anti-invasive factor during *B. pertussis* host infection (Bassinet *et al.*, 2000).

CyaA subverts T cell responses by DC modulation – Publication 4

CyaA of *B. pertussis* was shown to account for the suppression of monocyte-induced $CD4^+$ T cell proliferation in response to tetanus toxoid (Boschwitz *et al.*, 1997). Similarly, CyaA of closely related *B. bronchiseptica* and its type III secretion effectors impaired macrophage-induced $CD4^+$ T cell proliferation in response to OVA peptide. In contrast, no effect of *B. bronchiseptica*-pulsed DCs on inhibition of $CD4^+$ T cell proliferation was observed in concurrent experiments (Siciliano *et al.*, 2006).

In order to analyze whether CyaA of *B. pertussis* impairs T cell priming via modulation of DCs, which are the key antigen presenting cells of the immune system, we established an *in vitro* system of ovalbumin presentation to MHC class I and II-restricted T cells by bone marrow DCs. We have shown that CyaA impairs by 40 - 50 % the ability of LPS-stimulated DCs to activate OVA-specific T cell lines, measured as IL-2 production as well as to stimulate primary OT-I and OT-II T cells, determined as their proliferation.

This immunomodulatory action of CyaA on DCs *in vitro* could result from: (i) induction of DC death, comprising apoptosis or necrosis, (i) alteration of costimulatory molecule expresssion, (ii) modulation of cytokine secretion, (iii) inhibition of ovalbumin uptake and/or processing, and finally (vi) induction of apoptosis in interacting T cells. We could exclude that the observed effects were due to induction of DC death by CyaA, or induction of T cell apoptosis via DCs. The low concentration of CyaA (10 ng/ml) used in our experimental system even prevented LPS-induced apoptosis of DCs, consistent with the report showing suppression of spontaneous apoptosis of neutrophils by cAMP signaling (Kato *et al.*, 2006). We could exclude also the effects of CyaA on inhibition of ovalbumin uptake and ovalbumin processing, respectively. It is, however, important to note that CyaA suppressed DC macropinocytosis, in accordance with our previous study, showing macropinocytosis arrest in macrophages (Publication 3). Indeed, inhibition of

macropinocytosis *in vivo* upon CyaA encounter might contribute to decrease in antigen uptake and a lower T cell-stimulatory capacity of DCs (von Delwig *et al.*, 2002; von Delwig *et al.*, 2006). Here, however, ovalbumin was internalized predominantly via mannose receptor-mediated endocytosis, and not through macropinocytosis.

In contrast to that, in our *in vitro* experimental system, cAMP signaling of CyaA altered maturation and cytokine production profile of LPS-stimulated DCs. We dissected these alterations by using Epac-specific (8-pCPT-cAMP) and PKA-specific (6-Bnz-cAMP) cAMP analogs (Enserink *et al.*, 2002; Christensen *et al.*, 2003), and showed that solely activation of PKA appeared to mediate CyaA-induced suppression of LPS-stimulated CD40 and CD54 molecule expression, and enhancement of IL-10 cytokine production. In turn, CyaA signaling through Epac, as well as PKA, inhibited upregulation of H-2K^b molecules, and TNF-α and IL-12p70 cytokine production. We further revealed that CyaA signaling through PKA, and not through Epac, impaired DC capacities to stimulate ovalbumin-specific T cell proliferation *in vitro.*

Based on these observations, we propose that inhibition of T cell stimulation upon DC treatment with CyaA was primarily due to a combined effect of enhanced IL-10 secretion and decreased expression of CD40 and CD54 costimulatory molecules. Nevertheless, production of other soluble factors, such as prostaglandins (Siciliano *et al.*, 2006) and/or expression of other regulatory surface molecules (Brown *et al.*, 2003; Crow, 2006) might also have contributed to the observed inhibition.

Importantly, we also demonstrated here that when LPS-stimulated and OVA-peptide loaded DCs are treated by CyaA *ex vivo* and injected into mice, their ability to activate the expansion of adoptively transferred ovalbumin-specific CD8$^+$ T cells *in vivo* is impaired. This might rely on the same mechanisms as impairment of T cell proliferation *in vitro*. Alteration of DC migration and/or induction of Treg by CyaA-treated DCs *in vivo* may however also contribute to the observed outcome. Further experiments are needed to address these hypotheses.

We propose that action of CyaA on DCs might represent an additional strategy of *Bordetella* to attenuate adaptive immune defences at the site of colonization and delay the development of systemic immune response, thereby contributing to pathogen survival on the respiratory epithelia. The inhibition of T cell responses has, indeed, been documented in the context of *B. pertussis* infection. T cells isolated from lungs of infected mice were hyporesponsive to purified *B. pertussis* antigens and exhibited a decreased CD28 expression (McGuirk *et al.*, 1998). CyaA would act here in synergy with other *Bordetella* virulence

factors, such as FHA (Boschwitz *et al.*, 1997; Carbonetti, 2007; McGuirk *et al.*, 2002).

The observation that CyaA-targeted DCs have decreased ability to prime $CD8^+$ T cells might also be relevant in the context of *B. pertussis* infection. Albeit $CD8^+$ T cells do not seem to be crucial for the establishment of protective immunity against *Bordetella* (Leef *et al.*, 2000; Mills *et al.*, 1993), they might contribute to the defence against secondary infections, thereby very likely playing a role in the course of *Bordetella* infection and the recovery from the whooping cough.

Part II.: Use of adenylate cyclase toxoid for antigen delivery.

Within the second part of the PhD. thesis, we have exploited the capacity of CyaA toxoids to induce $CD8^+$ T cell responses in experimental model of rodent malaria, aiming to confer protection against challenge with *Plasmodium berghei* sporozoites.

Malaria currently ranks among the most prevalent infections in tropical and sub-tropical areas throughout the world. Every year, there are 350-500 million cases of malaria and the disease kills more than one million people, mainly African children. The increased incidence of malaria in many countries, caused by drug-resistant parasites (*Plasmodium falciparum* and *P. vivax*) and insecticide-resistant vectors (*Anopheles* mosquitoes), highlight the need to develop new methods of controlling this disease (Tsuji *et al.*, 2001).

Experimental vaccination with radiation-attenuated *Plasmodium* sporozoites can produce a solid sterile immunity that protects against the disease. This was first shown in studies with mice and later with human volunteers, demonstrating the feasibility of developing an effective malaria vaccine. However, vaccines based on radiation-attenuated sporozoites are not feasible for large scale application due to lack of *in vitro* culture system. Therefore, the development of peptide-based subunit vaccines has been undertaken as an alternative approach (Tsuji *et al.*, 2001; Vanderberg, 2009).

The complex life cycle of *Plasmodium* begins when a female mosquito, taking a blood meal, injects sporozoites into the blood. The sporozoites enter the bloodstream and within less than 30 min migrate to the liver and invade hepatocytes, where they mature over a period of 6–16 days. This period, known as “pre-erythrocytic stage” ends with the release of tens of thousands of merozoites, invading RBCs in the bloodstream. Within the RBCs, merozoites multiply and undergo a trophozoite and a schizont stage, which eventually bursts, lysing the RBC and releasing new generation of merozoites. These merozoites immediately proceed to invade new RBCs to repeat the cycle. This “intra-erythrocytic stage” of the disease is characterized by the classical acute febrile episodes that occur every 48–72 h, in coincidence with the synchronized lysis of infected RBCs and the release of newly matured merozoites. Some of the merozoites eventually develop into “sexual-stage” gametocytes, which, after being taken up by an anopheline mosquito, mature and sexually combine to generate a zygote, giving rise to new sporozoites, which are ready to reinitiate the cycle (Girard *et al.*, 2007).

Development of malaria vaccines has focused on the targeting of different stages of

parasite development, the pre-erythrocytic, the intra-erythrocytic, or the sexual stage, respectively. The pre-erythrocytic vaccine strategies aim to protect against malaria infection, and should provide humoral immunity by eliciting neutralizing antibodies that target the sporozoites and prevent their invasion into the liver. As an alternative, these vaccines should induce a cell-mediated immune response able to interfere with multiplication cycle of the parasites within the liver, e.g. by killing the parasite-infected hepatocytes (Girard *et al.*, 2007). Recombinant proteins and/or synthetic peptides containing defined B and T-cell epitopes of different antigens expressed in sporozoites and/or liver stages have been used as pre-erythrocytic malaria vaccines in experimental animal models. They have been shown to be highly immunogenic and capable of inducing protective immunity mediated by antibodies, as well as $CD4^+$ and IFN-γ secreting $CD8^+$ T-cells (Girard *et al.*, 2007; Hoffman and Doolan, 2000; Tsuji *et al.*, 2001). Nevertheless, other malaria vaccine strategies focus on the intra-erythrocytic stage, inhibiting parasite RBC invasion cycles and aim to protect against the severity of the disease, as well as on the sexual stage of the parasite, aiming to prevent transmission of the parasite to new hosts (Girard *et al.*, 2007).

In general, cytotoxic $CD8^+$ T cells (CTLs) represent one of the major and essential host defense machinery in the prevention and/or treatment of a number of disease states, including cancer and infections with intracellular pathogens. To induce CTLs, naive $CD8^+$ T cells must be primed by presentation of short antigenic peptides (usually derived from endogenous, cytosolic antigens) on MHC class I molecules on surface of APCs that also provide costimulatory signals. It is thus generally considered that the development of vaccines capable of stimulating CTLs requires the synthesis or delivery of the antigen in the cell cytosol of APCs (Moron *et al.*, 2004).

Some of the strategies currently exploited for CTL activation and MHC class I delivery are based on the use of DNA vaccines: (i) naked DNA vectors, (ii) replication-defective viruses (e.g. modified vaccinia virus Ankara, adenoviruses), or (iii) live attenuated bacterial vectors (e.g. Bacille Calmette–Guerin (BCG), *Listeria monocytogenes*, *Salmonellae*, *Shigellae)*; (Amara *et al.*, 2001; Shata *et al.*, 2000). This approach, however represent a safety risk because oncogenic DNA integration into the host genome cannot be excluded. Also genetic recombination of live attenuated vectors with other infectious agents and their reversion to virulent strains can occur. Alternative possibility to deliver antigens to the MHC class I presentation pathway is exploitation of the cell cross-presentation pathway by the use of certain adjuvants, synthetic lipopeptides, pH sensitive liposomes, immune complexes, heat-

shock proteins, virus-like particules, apoptotic cells and many other particulate antigens (Goletz *et al.*, 1997; Moron *et al.*, 2004; Simsova *et al.*, 2004).

Bacterial protein toxins which exert their toxic effect by modifying cytosolic components and possess the cell cytosol translocating capability, however, also appear to be ideally suited for the delivery of heterologous CTL epitopes in the class I presentation pathway, provided that detoxification without apparent loss of delivery capability can be achieved. Many of these toxins, including exotoxin A of *Pseudomonas aeruginosa*, Shiga-like toxin 1 and heat-labile enterotoxin of *E. coli*, Shiga toxin of *Shigella dysentariae,* diphtheria toxin of *Corynebacterium diphtheria*, anthrax toxin of *Bacillus anthracis* and pertussis and adenylate cyclase toxin of *Bordetella pertussis* have been tested for delivery of antigens into cell cytosol. They have been genetically engineered to carry $CD8^+$ T-cell epitopes, and shown *in vitro* to deliver antigens into the class I presentation pathway. Nevertheless, they do not convincingly induce specific CTL responses *in vivo,* with exceptions of *Bordetella* CyaA and Shiga toxin of *Shigella dysentariae* (Fayolle *et al.*, 1996; Haicheur *et al.*, 2000; Moron *et al.*, 2004; Saron *et al.*, 1997).

Towards the aim of constructing a vaccine against *Plasmodium*, we went on to demonstrate the versatility of the detoxified CyaA vector (unable to form cAMP) for heterologous epitope delivery and induction of CTL responses *in vivo.* We employed previously defined, pre-erythrocytic stage malaria antigen, circumsporozoite protein (CSP) and experimental mice malaria model (Romero *et al.*, 1989), aiming to confer protection against challenge with *P. berghei* sporozoites.

Currently, various efforts in the field of malaria vaccinology has been undertaken to maximize immunogenicity of the chosen antigen, employing either new vaccine carriers or new adjuvants (Girard *et al.*, 2007). A main objective of these strategies is the ease of application and the capacity of the vaccine to target antigens into the MHC class I presentation pathway, as a prerequisite for the effective induction of $CD8^+$ T cells. Indeed, the intracellular hepatic stage of the *Plasmodium* life cycle is an ideal target for protective immune response, because this stage lasts for at least 6 days and is not associated with pathology. A vaccine that is able to effectively target the infected hepatocyte would prevent both the clinical symptoms of malaria, which first arise during the erythrocytic stage, and the transmission of malaria, since fertilization occurs during the sexual stage (Engwerda and Good, 2005; Hoffman and Doolan, 2000).

Protection against *P. berghei* liver-stage malaria – Publication 5

We constructed, here, the recombinant CyaA toxoid carrying CSP-derived $CD8^+$ T-cell epitope SYIPSAEKI, presented on $H\text{-}2K^d$ and, as a marker, the OVA-derived SIINFEKL epitope presented in the context of $H\text{-}2K^b$. The inserted sequence, harboring the $CD8^+$ T-cell epitope SYIPSAEKI, corresponding to residues 245 to 253 of CSP, contained also the natural flanking sequences of the epitope, to ensure appropriate processing by the proteasome. We also introduced additional positively charged amino acid residues toward the termini of the inserted sequence to compensate for the negatively charged amino acid residues present in the insert, as insertion of negative charges within the AC domain was reported to inhibit the CyaA penetration (Karimova *et al.*, 1998a). By using the OVA tag, we were able to show that the resulting recombinant CyaA-CSP toxoid delivered the AC domain into the MHC class I presentation pathway both, *in vitro* as well as *in vivo*. We further detected by MHC multimer staining induction of CSP-specific $CD8^+$ T cells in the spleens, blood and livers of immunized mice, which upon *ex vivo* restimulation with CSP peptide secreted IFN-γ. The induction of the specific CTL response *in vivo* by the use of CyaA vector, targeted to antigen-presenting cells via CD11b/CD18 interaction, is in agreement with previously published data (Dadaglio *et al.*, 2003; Fayolle *et al.*, 2004; Fayolle *et al.*, 1996).

Indeed, the efficacy of the bacterial toxins to stimulate naive $CD8^+$ T cells *in vivo* could be related to their capacity to target DCs. Receptors of the anthrax toxin, pertussis toxin and exotoxin A are expressed on a wide variety of eukaryotic cells that could represent a disadvantage for inducing efficient CTL responses *in vivo* (Moron *et al.*, 2004). By contrast, cell invasion by the Shiga toxin depends on the interaction of its B subunit with the globotriaosylceramide Gb3 receptor, being express by various DC types. Interestingly, Shiga toxin in addition to the CyaA toxin, is one of the few systems for which convincing evidence of *in vivo* CTL activation against tumor antigens has been obtained (Haicheur *et al.*, 2000; Moron *et al.*, 2004).

The main aim of any vaccine is, however, not only to trigger strong CTL responses but more importantly to elicit protective and/or therapeutic responses. Although single administration of CyaA-CSP toxoid induced the high numbers of CSP-specific $CD8^+$ T cells, no protection against subsequent challenge with *P. berghei* sporozoites was achieved. As protection against malaria is achievable by INF-γ-producing MHC class I restricted $CD8^+$ T cells (Engwerda and Good, 2005; Tao *et al.*, 2005), this failure might be explained by the special status of the liver, which is known to dampen cytokine production by T cells or to induce their anergy (Tiegs and Lohse, 2009).

A potent negative regulator of the immune response, whose manipulation became a valuable target for immunological therapies, is a CTLA-4 molecule, being expressed on activated T cells and T regulatory (Treg) cells (Martins *et al.*, 2004; Sarnaik and Weber, 2009). When the CyaA-CSP was administered in combination with anti-CTLA-4 Ab during the boost immunization, the frequencies of the induced CSP-specific T cells were superior to those induced by the CyaA-CSP toxoid alone. This resulted into a statistically significant degree of protection upon a sporozoite challenge, with 60% of the animals being fully protected and not developing the parasitemia.

Interestingly, the blockade of CTLA-4 had a major impact during the booster immunization with CyaA-CSP and not on the efficacy of the primary immunization with the toxoid. When anti-CTLA-4 was administered during the primary immunization, no statistically significant increase in the number of IFN-γ-producing T cells was observed, compared to the administration of CyaA-CSP alone. This demonstrates, that counterregulation of the immune system following the primary immunization dampens the immune response elicited by the vaccine. The immunization with CyaA-CSP toxoid, however, did not increase the frequency of $Foxp3^+$ Treg cells. Therefore, the expansion of vaccine-induced T cells might be limited either by the pool of naturally occurring Treg cells, without an increase in frequency, or by induction of CTLA-4 expression on T effector or memory cells during the primary immunization. In both cases, temporary blockade of the regulatory pathway during a booster immunization would lead to an increase in the activation of T cells and hence would represent a promising way to potentiate vaccine-induced T cell responses.

A major drawback that dampens the use of anti-CTLA-4 Ab is the risk of inducing severe inflammatory side effects, such as those observed in clinical trials of cancer immunotherapy (Blansfield *et al.*, 2005). However, in the course of vaccine administration, the harmful side effects might be controllable, as CTLA-4 blockade has to be achieved only for a very short time during booster immunization, compared to the repeated administration of the anti-CTLA-4 Ab in immunotherapy of tumors.

Improvements of the CyaA vaccination strategy against sporozoite challenge are also feasible. CyaA toxoids carrying three different epitopes, including a LCMV epitope, were previously shown to induce specific CTL responses *in vivo* for each of the three epitopes, as well as to protect against a lethal LCMV challenge (Fayolle *et al.*, 2001). Therefore, insertion of various $CD8^+$ T cell epitopes of liver stage *Plasmodium* parasites into the same CyaA recombinant molecule would be plausible. Also, combination of several immunodominant T cell epitopes resulting into a sequential immunological assault against the malaria parasite as

it progresses through its different developmental stages and generation of a multi-stage vaccine could be considered (Vanderberg, 2009). Finally, as protective immune response against malaria parasites relies also on $CD4^+$ T cells (Engwerda and Good, 2005) and CyaA toxoids are able to reach both MHC class I and II antigen presentation pathway (Loucka *et al.*, 2002; Schlecht *et al.*, 2004), insertion of $CD4^+$ T cell epitopes, and activation of $CD4^+$ T-helper cells in parallel to induction of $CD8^+$ T cytotoxic cells could be of interest.

Complete protection against *P. berghei* liver-stage malaria – Publication 6

Nevertheless, up to now the most promising vaccine strategies rely on heterologous prime/boost immunization in which two different strategies to induce $CD8^+$ T cells are combined (Gilbert *et al.*, 2002; Lu, 2009). Therefore, we examined the vaccine potency of CyaA-CSP toxoid in a heterologous prime/boost immunization regimen with live attenuated *Salmonella typhimurium* vaccine*,* expressing the YopE-CSP fusion. It was shown previously that the YopE (*Yersinia* outer protein E) translocation domain can be employed as carrier domain for cytosolic antigen delivery by the *Salmonella* type III secretion system, and that recombinant *Salmonella* expressing fusions of YopE with different antigens elicit specific MHC class I-restricted T cell responses in orally vaccinated mice (Flynn *et al.*, 1990; Russmann, 2003; Russmann *et al.*, 1998). We have shown here, that oral vaccination with recombinant *Salmonella* strain, expressing the YopE-CSP fusion, resulted in the induction of CSP-specific IFN-γ-producing $CD8^+$ T cells in spleen and liver, which upon a heterologous boost immunization with CyaA-CSP yielded a sterile protection against a sporozoite challenge in all vaccinated mice.

Interestingly, the reversed order of vaccine administration (CyaA-CSP as prime and *Salmonella* as boost) led to a reduced response. This might be due to activation of regulatory mechanisms that are often associated with infectious agents and would dampen the efficacy of *Salmonella* as a booster vaccine. In this respect, CyaA appears to be a much more promising carrier for a booster vaccine, since no increase in the number of $Foxp3^+$ Treg cells was observed upon repeated application of the CyaA-CSP toxoid. Due to these characteristics CyaA is a promising vaccine carrier to boost the efficacy of malaria vaccines that are already used in clinical trials.

CONCLUSIONS

Part I.: Molecular mechanisms of action of the adenylate cyclase toxin

CyaA as a swift saboteur of host defense - Publication 1

- Significant progress has been made in understanding of the interaction of CyaA with target cells. In this review we highlighted the most important findings in the past five years.
- These are: description of an integrin 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, is effective in elevating intracellular cAMP levels.
- New focus of research on CyaA is the potential of the toxin to exert immunomodulatory activity through action on dendritic cells and epithelial cells.

Penetration of AC domain of CyaA across cell plasma membrane – Publication 2

- We showed that the first 371 amino-terminal residues are dispensable for the CyaA capacity to deliver a passenger OVA epitope into cytosol of dendritic cells, determined as *in vitro* stimulation of OVA-specific $CD8^+$ T cells, and hence membrane penetration of the CyaA.
- We suggested that AC domain participates in membrane penetration only a passive passenger, a cargo.

cAMP signaling of CyaA subverts phagocyte function - Publication 3

- Using pull-down activity assays, we found that CyaA-catalyzed elevation of cytosolic cAMP in murine macrophages causes transient and selective inactivation of RhoA, in the absence of detectable activation of Rac1, Rac2 or RhoG.
- This CyaA/cAMP-induced drop of RhoA activity in macrophages yielded massive actin cytoskeleton rearrangements and formation of transient sheet-like membrane ruffles, the formation of which further depended on Rac1 activity, as determined by transfections with mutant Rho family GTPases.
- We further showed that CyaA-induced mebrane ruffling is futile and is accompanied by an arrest of macropinocytosis and a rapid and complete block of CR3-mediated phagocytosis.
- We report that the repeatedly documented capacity of CyaA to undermine bactericidal activities of macrophages may well rely on RhoA inactivation as a result of cAMP signaling.

CyaA subverts T cell responses by DC modulation - Publication 4

- We examined the ability of CyaA to modulate LPS-induced maturation of bone marrow DCs from mice and their T cell priming capacities and determined the respective contributions of PKA and Epac-signaling to these processes.
- By using PKA- and Epac-specific cAMP analogs, we demonstrated that cAMP signaling of CyaA modulated LPS-stimulated maturation of DCs through PKA as well as Epac signaling. Solely PKA activation appeared to mediate suppression of LPS-induced CD40 and CD54 molecule expression, and enhancement of IL-10 cytokine production, while Epac as well as PKA signaling inhibited LPS-induced upregulation of H-2K^b molecules, and TNF-α and IL-12p70 cytokine production.
- We further showed that CyaA signaling through PKA, and not through Epac, impaired DC capacities to stimulate OVA-specific CD4$^+$ and CD8$^+$ T cell proliferation *in vitro*.
- Using adoptive transfer, we determined that CyaA-treated DCs also impaired expansion of CD8$^+$ T cells *in vivo*.

Part II.: Use of adenylate cyclase toxoid for antigen delivery

Protection against *P. berhei* liver-stage malaria - Publication 5

- After a single intraperitoneal application of the CyaA-CSP toxoid, we detected induction of a CSP-specific $CD8^+$ T cells by MHC multimer staining and IFN-γ ELISPOT assay in immunized mice, while no protective immunity against challenge with *P. berghei* sporozoites was achieved.
- Administration of anti-CTLA-4 during boost immunization led to a significant enhancement of the CSP-specific $CD8^+$ T cells and induced a protective immunity against subsequent challenge with *P. berghei* sporozoites.

Complete protection against *P. berghei* liver-stage malaria - Publication 6

- We employed here oral immunization with an attenuated live *Salmonella* vaccine, delivering the $CD8^+$ T-cell epitope of circumsporozoite protein (CSP) through type III secretion system, as a primary vaccine strategy. As a booster vaccine carrier, we used the CyaA toxoid bearing the same CSP epitope.
- This heterologous prime/boost vaccination regimen conferred sterile immunity against subsequent challenge with *P. berghei* sporozoites in all vaccinated mice.
- We suggested CyaA to be a promising vaccine carrier to boost efficacy of vaccines in heterologous prime/boost immunizations.

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