Immunomodulation of dendritic cells by adenylate cyclase toxin from *B. pertussis*

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Abstrakt

*Bordetella pertussis* je gramnegativní bakterie způsobující onemocnění dýchacích cest černý kašel, které může skončit až smrtí především u dětí a novorozenčů. *B. pertussis* produkuje několik toxinů, mezi které patří i adenylát cyklážový toxin (CyaA). CyaA je důležitým patogenním faktorem, jelikož bylo ukázáno, že bakterie *B. pertussis* s deletovaným CyaA nebyly schopny kolonizovat plice a vyvolat infekci v myším modelu. CyaA se váže s vysokou afinitou na CD11b/CD18 integrin vyskytující se na makrofáziích, neutrofílech, NK buňkách a dendritických buňkách (DCs), kde po translokaci do cytosolu katalyzuje konverzi ATP na suprafyziologické množství cAMP. Dendritické buňky jsou nejdůležitější buňky prezentující antigeny, které stimuluji T buněčnou adaptivní odpověď. Bylo ukázáno, že CyaA moduluje spektrum sekretovaných cytokinin u DCs stimulovaných LPS. Dále byl popsán vliv CyaA na maturaci DCs tím, že mění expresi kostimulačních a adhezních molekul. V naší laboratoři bylo zjištěno, že CyaA inhibuje prezentaci na MHC molekulách I a II třídy, a tím potlačuje stimulaci CD8+ a CD4+ T buněk. Nedávno jsme pozorovali, že CyaA inhibuje makropinocytózu u DCs, což je stejně jako v případě inhibice T buněk, dáně jeho schopností tvořit cAMP. O možném účinku CyaA na přijem antigenu a jeho zpracování DCs toho není příliš známo. Vzhledem k tomu, že byl v buňkách imunitního systému popsán negativní vliv cAMP signalizace na zpracovávání antigenu, můžeme předpokládat, že by CyaA mohl tyto procesy také ovlivnit v DCs.

**Klíčová slova:** adenylát cyklážový toxin; cAMP; dendritické buňky; T buňky; antigenní prezentace; *Bordetella pertussis*
Abstract

*Bordetella pertussis* is the causative agent of whooping cough, a respiratory disease representing a severe and life-threatening illness, particularly in infants and children. *B. pertussis* produces a wide range of toxins, including adenylate cyclase toxin (CyaA). CyaA is an important pathogenic factor as it was shown that CyaA-deficient bacteria are not able to colonize lungs and establish persistent infection in mouse model. CyaA binds with a high affinity to the CD11b/CD18 integrin on macrophages, neutrophils, natural killer cells and dendritic cells (DCs) and generates supraphysiological levels of cAMP in the cytosol of host cells. DCs are the most important antigen-presenting cells for stimulation of T cell adaptive responses. It was shown that CyaA modulates the secretion of cytokines in LPS-stimulated DCs and drives them into a semi-mature state. In our laboratory, we found that CyaA inhibits MHC class I and MHC class II presentation by bone marrow-derived dendritic cells (BMDCs), thereby impairing stimulation of CD8⁺ and CD4⁺ T cells, respectively. Recently, we have observed that CyaA inhibits macropinocytosis in DCs which was also dependent on its ability to generate cAMP. Little is known about the possible impact of CyaA on antigen uptake and processing in DCs. As negative effects of cAMP signalling on antigen processing have been described in immune cells, it is possible that CyaA might affect these processes in DCs as well.

**Key words:** adenylate cyclase toxin; cAMP; dendritic cells; T cells; antigen presentation; *Bordetella pertussis*
Introduction

*Bordetella pertussis* is the causative agent of whooping cough a disease that affects 20- to 40-million people worldwide each year and causes some 200,000 to 400,000 fatalities (WHO [http://www.who.int]). Despite the fact that an efficient immunization with a considerably improved safety profile has been already found, developing countries, where it is most needed, cannot afford it. *B. pertussis* produces a wide range of toxins, including adenylate cyclase (CyaA). CyaA plays an important role in early stages of respiratory tract colonization and in the establishment of the infection (Goodwin and Weiss, 1990). CyaA binds with a high affinity to the CD11b/CD18 integrin (Gueronprez et al., 2001) found on macrophages, natural killer cells and dendritic cells (DCs), which most likely represent the primary target cells in vivo and generates high levels of intracellular cAMP, an important second messenger.

My work is connected to one of the research topics of the Laboratory of Molecular Biology of Bacterial Pathogens in Institute of Microbiology of the Czech Academy of Sciences, where the research has been mainly focused on CyaA biochemistry and its interaction with the cells of innate immunity. It should be mentioned here that it has not been until very recently that the laboratory started to focus on modulation of DCs by CyaA.

In the first part, my work deals with DCs and T cell biology. DCs are very important antigen-presenting cells in immune system and their immunomodulation reflects on activation and differentiation of T cells, thereby determining the character of immune response. The second part of this work summarizes the current knowledge about CyaA and its interaction with DCs. CyaA-generated cAMP was demonstrated to modulate the production of cytokines in LPS-induced DCs and to drive DCs into semi-mature state by influencing the expression of MHC class I and II and costimulatory molecules (Bagley et al., 2002; Boyd et al., 2005; Ross et al., 2004; Skinner et al., 2004; Spensieri et al., 2006). However, little is known about the effects of CyaA on the capacity of DCs to stimulate T cell adaptive responses. In our laboratory, we found that CyaA inhibits stimulation of CD8⁺ and CD4⁺ T cells. In last chapters, I discuss the possible effects of CyaA on antigen (Ag) uptake and processing as it was documented that Ag processing can be negatively influenced by high intracellular cAMP levels (Matousek et al., 1998; Matousek et al., 1996). Moreover, the inhibition of Ag presentation as a strategy of many other pathogenic bacteria is also delineated. Whether the inhibition of Ag processing might be one of the mechanisms by which CyaA affects Ag presenting ability of DCs is a question for which I will seek to find an answer in my future work.
1. Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) which have a very important role in innate as well as in adaptive immunity (Banchereau et al., 2000). They are considered to be the most efficient APCs. Immature DCs with a high phagocytic and endocytic capacity are located in peripheral tissues where they capture and process antigens (Ags). After maturation they migrate into secondary lymphoid organs and present the collected Ags to T cells to initiate adaptive immune responses. They not only activate, but also tolerize T cells to self-antigens, thereby minimizing autoimmune reactions and maintaining peripheral tolerance.

1.1 Maturation of DCs

The term maturation refers to a complex differentiation process whereby DCs answer to an environmental stimulus and become capable of inducing adaptive immunity. Maturation is initiated in the periphery by a number of factors including pathogen-related molecules (e.g. LPS, double-stranded RNA, bacterial DNA), various pro-inflammatory and inflammatory signals (e.g. TNF-α, prostaglandins) or T cell-derived signals (e.g. CD40L) (Banchereau et al., 2000). Also innate lymphocytes like natural killer (NK) cells, natural killer T (NKT) cells or γδ T cells can trigger DC’s maturation (Munz et al., 2005). DCs change morphologically which is manifested by a loss of adhesive structures, cytoskeleton reorganization and acquisition of a high cellular motility. Furthermore, they up-regulate the expression of costimulatory and adhesion molecules, secrete cytokines and chemokines, enhance antigen processing and presentation, and migrate to secondary lymphoid organs (Fig. 1).

![Immature DC vs Mature DC](image)

**Fig. 1.** Maturation of DCs (Banchereau et al., 2000; Verdijsk et al., 2004).
1.2 Subtypes of DCs

DCs arise from both myeloid and lymphoid progenitors within the bone marrow. There are two main classes of DCs: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). pDC generate large amounts of type I interferons and are particularly involved in response to viral infections (Colonna et al., 2004). cDCs differ in their phenotype, localization and function and are involved mainly in Ag presentation and activation of naïve T cells. All murine cDCs express CD11c integrin on their cell surface. Moreover, additional subtypes can be distinguished according to the expression of other cell surface markers like CD4, CD8, CD11b or CD205. Spleen contains e.g. CD4⁺CD8⁺, CD4⁺CD8⁻ and CD4⁺CD8⁻ DCs. Other DC's subtypes involve CD4⁺CD8⁺CD11b⁺ DCs which are believed to be mature form of tissue interstitial DCs or Langerhans DCs found only in skin-draining lymph nodes which express high levels of langerin and E-cadherin. cDCs are located virtually in all tissues and organs, where they might exert specific functions. Even though different subtypes of mouse cDCs share a common capacity to present Ags to T cells, they might differ in the other functional aspects e.g. indoleamine 2,3-dioxygenase (IDO)-expressing DCs (Munn, 2002) or interferon-producing killer DCs (IKDCs) (Chan et al., 2006). In contrast to murine DC's subsets, relatively little is known about subsets of human DCs in vivo. Most of the insight into human DC's subtypes was obtained from studies of their development from peripheral blood monocytes in vitro. DCs can be generated also from bone-marrow by various growth factors and cytokines e.g. GM-CSF, IL-4, IL-3 (Shortman and Liu, 2002).

2. Antigen presentation

2.1 Antigen uptake by DCs

DCs capture Ags mainly by receptor-mediated endocytosis, macropinocytosis and phagocytosis (Watts and Amigorena, 2000). Receptor-mediated endocytosis requires receptors that are expressed on DCs, such as Fc receptors or members of the C-type lectin family. Macropinocytosis allows the internalization of non-specific fluid-phase solutes which leads to the formation of large vesicles. Unlike receptor-mediated endocytosis, macropinocytosis is down-regulated upon maturation in DCs (Sallusto et al., 1995). Phagocytosis encompasses the engulfment of solid particles like opsonized bacteria, viruses or apoptotic and necrotic cell fragments via Fc-, complement and various scavenger receptors to form a phagosome. Although phagocytosis of bacteria is not as efficient in DCs as it is
probably in macrophages, it leads not only to presentation of bacterial Ags, but also drives DC's maturation (Rescigno et al., 1998; Watts and Amigorena, 2000).

2.2 Antigen processing and presentation

T cells by their T cell antigen receptors (TCRs) must recognize peptide fragments of Ags bound to molecules of the major histocompatibility complex (MHC) on the surface of APC in order to initiate adaptive immunity. There are two types of MHC molecules: MHC class I which is recognized by CD8\(^+\) T cells to generate cytotoxic T cells and MHC class II which is recognized by CD4\(^+\) T cells to generate helper T cell responses.

2.2.1 MHC class I-restricted antigen presentation

MHC class I molecules display on their surface short peptide fragments derived from proteins synthesized by the cell. In cytosol, proteins are ubiquitinylated and directed into proteasome. Proteasome is formed by a 20S catalytic core complex and two 19S regulator protein complex that are responsible for the binding, unfolding and cleavage of ubiquitinated substrates. In immune cells there can be two types of proteasomes: proteasome that is constitutively expressed and immunoproteasome that is formed upon exposure to inflammatory mediators like IFN-\(\gamma\). IFN-\(\gamma\) induces formation of new protein subunits (PA28) that are incorporated into nascent 20S proteasome, thereby enhancing its processing capacity and MHC class I presentation (Strehl et al., 2005). Peptides can be further trimmed by cytosolic peptidases on their way to endoplasmatic reticulum (ER). Newly synthesized MHC class I molecules also assemble in ER with the help of a chaperone protein, calnexin. MHC class I molecule then binds to the peptide transporter-associated protein (TAP) tapasin which forms a bridge between MHC class I molecules and TAP1/2 transmembrane transporter. Together with calreticulin and Erp57, MHC class I molecule and tapasin form MHC class I loading complex. Erp57 and calreticulin are essential to maintain the MHC class I molecule in a state receptive to peptide and also carry out a peptide-editing function. Peptides are translocated into the ER via ATP-dependent TAP1/2 and are trimmed by the ER-resident amino peptidases to 8-10 amino acids. Finely, they are loaded onto MHC class I molecules and transported from ER through Golgi apparatus to the cell surface.

Peptides derived from endogenous proteins can be also presented on MHC class I molecules by a process called cross-presentation. DCs particularly were shown to be very efficient in cross-presentation (Albert et al., 1998). To date, there are no distinct entry routes known to deliver Ags preferentially for cross presentation (Monu and Trombeta, 2007), however peptides are believed to be transported into the cytosol from an endocytic compartment.
2.2.2 MHC class II-restricted antigen presentation

MHC class II molecules are associated with foreign peptides generated from proteins in the endocytic pathway. Proteins that enter cells through endocytosis are delivered to early endosomes (pH 6.1), which fuse with late endosomes and become increasingly acidic (pH 5.5). Late endosomes eventually fuse with lysosomes (pH 4.5). Protein processing into peptide fragments is carried out by various proteases e.g. cathepsins, IFN-γ-induced lysosomal thiol reductase (GILT) or lipases. Cathepsins, that play a major role in generating antigenic peptides, are synthesized as inactive precursors and become enzymatically active after the proteolytical cleavage in the presence of acidic pH (4.5). Their cysteine protease activity is also regulated by small-molecule inhibitors, such as cystatins (Honey and Rudensky, 2003).

MHC class II molecules assembly in ER with a polypeptide invariant chain (Ii) lying within the peptide-binding groove preventing so the unspecific peptide binding. Then the complex is transported through Golgi apparatus to a specific endocytic compartment MHC class II-rich compartments (MIICs) in the late endosomal pathway. In MIICs, Ii is cleaved by cathepsin S in DCs leaving a class II-associated invariant-chain peptide (CLIP) bound to MHC class II molecule. Immature DCs, specialized mainly in capturing Ags, contain high amount of preformed MHC class II molecules in MIICs and also express cystatin C which inhibits the action of cathepsin S. After maturation, the expression of cystatin C is downregulated and pH in endosomal pathway decreases thereby allowing the generation and loading of antigenic peptides (Riese et al., 1996). The loading is catalyzed by a MHC class II like molecule, HLA-DM/H-2M (human/mice) which ensures the removal of CLIP and also provides an editing of weakly bound peptides. Once loaded with peptides, MHC class II molecules are transported to the plasma membrane. The binding site of MHC class II molecule has open ends, thereby longer peptides (15-35 amino acids) can be presented.

2.3 T cells

T lymphocytes play a central role in controlling the adaptive immune response. They serve as crucial effector cells through antigen specific cytotoxic activity and the production of cytokines. Several different subtypes of T cells have been described based on their specific effector function: helper T cells (Th), cytotoxic T cells, memory T cells and regulatory T cells (also suppressor T cells; Treg) (Becker et al., 2006; Romagnani, 2006; Sallusto et al., 2004). Helper T cells expressing co-receptor CD4 are divided into Th1, Th2 and Th17 cells. Th1 cells are crucial for activating macrophages to eliminate intracellular bacteria and for providing help to B cells to produce antibody. They mainly produce IFN-γ, TNF-β, IL-3, and TNF-α. Th2 cells, that are the most effective activators of B cells, produce a different set of
cytokines, i.e. IL-4, IL-5, IL-9 and IL-13. Th17 cells help to recruit neutrophils to sites of infection, which is aimed mainly at extracellular pathogens. They release members of the IL-17 family such as IL-17A, IL-17E. Cytotoxic T cells (CTLs) express CD8 co-receptor on their surface. They provide protection against intracellular pathogens and are important in anti-tumour immunity. CTLs kill infected cells directly by forming pores by perforin and causing apoptosis either via secretion of granzymes or engagement of Fas receptor on the target cell. They also produce cytokines IFN-γ, TNF-α and LT-α, thereby contributing to macrophage activation. Memory T cells, CD4+ as well as CD8+, function as a dynamic reservoir of Ag experienced T lymphocytes that persist long-term after an infection has resolved. They can be divided into effector memory T cells (TEM) that migrate to inflamed peripheral tissues and display immediate effector function, and central memory T cells (TCM) that have little or no effector function, but proliferate to effector cells in response to antigenic stimulation (Sallusto et al., 2004). Regulatory T cells are represented by naturally occurring CD4+CD25+ regulatory T cells which develop in thymus and adaptive regulatory T cells which can be generated from conventional T cells in secondary lymph nodes. They actively control the properties of other immune cells by suppressing their functional activity to prevent autoimmunity, allergy and transplant rejection. They also play a negative role in cancer (Becker et al., 2006).

2.4 T cell activation

T cell activation occurs only if T cell antigen receptors (TCRs) recognize their specific ligands, MHC-peptide and CD28 engage costimulatory molecules on the surface of APC in the secondary lymphoid tissue. After initial adhesion, the immunological synapse is formed by polarizing adhesive and signalling molecules around the site of contact between a T cell and an APC (Grakoui et al., 1999). The contact surface of the T cell is organized into two zones on the mature synapse. Central zone is known as central supramolecular activation complex (cSMAC). It contains most of the proteins important in T cell activation (TCR, CD4 or CD8 co-receptors, MHC-peptide complexes, CD28, CD2 etc.). The outer zone is called peripheral supramolecular activation complex (pSMAC). This zone is enriched of adhesive molecules LFA-1 and VLA-4, talin or transferin receptor. Immunological synapse is considered to have an important role in regulation of T cell signalling. Whereas the activating and inhibitory molecular interactions in the immunological synapse last only seconds, the required duration of signalling, which is necessary for full T cell activation and proliferation, is in the order of hours (Bromley et al., 2001; Grakoui et al., 1999).
3. *Bordetella pertussis*

*Bordetella pertussis* is a highly efficient gram-negative pathogen, which is a causative agent of whooping cough. It is a strictly human pathogen, although it can infect other mammals as well. Bacteria colonize the mucosa of the upper respiratory tract and synthesize a variety of virulence factors, including adhesins and toxins (Mattoo and Cherry, 2005). Beside lipopolysaccharide (LPS) which differs in structure from that of *E. coli* (Caroff et al., 2001), the most important pathogenic factors are summarized in Table 1.

The illness usually begins with the most infectious catarrhal stage which is characterized by nonspecific symptoms such as cold, conjunctival irritation and slight cough. After 7 to 10 days, a characteristic cough appears and is followed by a whoop which in new-born children and very young infants may be replaced with apnea and cyanosis. In this age, the disease is most severe and life threatening because of secondary infections. Among immunized individuals, especially adolescents and adults, the disease is often mild and confused with other common causes of chronic cough such as asthma. Immunization with whole cell pertussis vaccines (Pw) protects against disease. However, its association with mild to severe neurological complications, led to a development of a new generation of acellular pertussis vaccines (Pa) which comprise purified antigenic components of *B. pertussis* and have considerably improved safety. However, despite intensive vaccination whooping cough still presents a major health problem in some part of the world.

The adaptive immunity of the host plays an important role in combating *B. pertussis* infection. Direct evidence of the importance of T cells in immunity to *B. pertussis* was provided by the demonstration that athymic or severe combined immunodeficient (SCID) mice failed to clear the bacteria, whereas normal BALB/c mice cleared it (Mills et al., 1993). Adoptive transfer experiments confirmed the protective role of T cells. *B. pertussis*-specific CD4⁺ T cells from mice primed by infection, but not CD8⁺ T cells, were shown to confer protection to athymic or sublethally irradiated recipient mice in the absence of detectable antibody responses (Lee et al., 2000). Further evidence of a role of CD4⁺ T cells was provided by the observation that CD4⁺ T cell knock out mice could not be protected by intranasal immunization with inactivated bacteria. In contrast, CD8⁺ T cells-depleted or β2 microglobulin knock out mice, which lack mature CD8⁺ T cells, mediated protection against *B. pertussis* (Lee et al., 2000). Taken together, these data suggest that CD4⁺ T cells, but not CD8⁺ T cells, mediated protection against *B. pertussis*. 
Table 1. Main pathogenic factors of *B. pertussis* (Mattoo and Cherry, 2005)

<table>
<thead>
<tr>
<th>Pathogenic factor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adhesins</strong></td>
<td></td>
</tr>
<tr>
<td>Filamentous hemagglutinin (FHA)</td>
<td>dominant adhesin, required for tracheal colonization, highly immunogenic, primary component of acellular pertussis vaccines</td>
</tr>
<tr>
<td>Fimbriae (FIM)</td>
<td>filamentous cell surface structures, required for persistent tracheal colonization primary component of acellular pertussis vaccines</td>
</tr>
<tr>
<td><strong>Autotransporters</strong></td>
<td></td>
</tr>
<tr>
<td>Pertactin (PRN)</td>
<td>surface protein, mediates eukaryotic cell binding in vitro, enhances protective immunity</td>
</tr>
<tr>
<td>BrkA</td>
<td>putative adhesin, confers serum resistance and protection against antimicrobial peptides</td>
</tr>
<tr>
<td><strong>Toxins</strong></td>
<td></td>
</tr>
<tr>
<td>Pertussis toxin (PT)</td>
<td>AB toxin, ADP-ribosylates G proteins, responsible for pertussis-associated lymphocytosis, strong adjuvant and primary component of pertussis vaccines</td>
</tr>
<tr>
<td>Adenylate cyclase (CyaA)</td>
<td>calmodulin-activated RTX family toxin with dual adenylate cyclase/hemolysin activity, acts as anti-inflammatory and anti-phagocytic factor during infection</td>
</tr>
<tr>
<td>Dermonecrotic toxin (DNT)</td>
<td>heat labile secreted toxin, activates Rho GTPases, induces necrosis in vitro</td>
</tr>
<tr>
<td>Tracheal cytotoxin (TCT)</td>
<td>causes mitochondrial bloating, disruption of tight junctions, damage to cilia, induces IL-1α and NO production in host cells</td>
</tr>
</tbody>
</table>

### 4. Adenylate cyclase toxin

Adenylate cyclase toxin (CyaA, ACT, AC toxin) produced by *B. pertussis* is a member of the RTX (repeat in toxin) family of bacterial pore-forming toxins. CyaA is an important pathogenic factor as it was shown that strain lacking CyaA was unable to colonize lungs and establish infection in mouse model (Goodwin and Weiss, 1990). CyaA is a 1706 amino acids long protein which has two functional domains. The C-terminal domain (~1300 amino acids) possesses membrane-targeting and pore-forming activity, whereas the N-terminal domain (~ 400 amino acids) carries an adenylate cyclase (AC) activity. The AC domain is translocated into the cytosol of the target cell. After binding of eukaryotic calmodulin, it catalyzes unregulated conversion of cellular ATP to cAMP, a key second messenger signalling molecule. The C-terminal domain, containing glycine and aspartate-rich repeats harbouring a conserved sequence motif X-(L/I/F)-X-G-G-X-G-(D/N)-D, has an intrinsic haemolytic activity that results from its ability to form cation-selective channels in the cell membrane (Ladant and Ullmann, 1999). CyaA becomes fully active after an acylation by the acyltransferase CyaC and binding of Ca\(^{2+}\) ions into RTX domain. It was shown, that acylation of Lys-983 seems to be crucial whereas acylation of Lys-860 is not necessary for CyaA activity (Masin et al., 2005). Acylation is important for a tight interaction of the toxin with its receptor CD11b/CD18 integrin on target cells (El-Azami-El-Idrissi et al., 2003).
Fig. 2. Adenylate-cyclase toxin of *B. pertussis* (Simsova M. et al., 2004).

### 4.1 Interaction of CyaA with cells of immune system

Even though CyaA is able to interact with a wide variety of eukaryotic cells, it was shown that this toxin binds to target cells specifically via CD11b/CD18 (also CR3, Mac-1) (Guermonprez et al., 2001). This member of the β2-integrin family is expressed on myeloid immune cells like macrophages, neutrophils, DCs or natural killer cells. These cells are predominantly targeted by CyaA in vivo as it was demonstrated that CyaA of *B. pertussis* inhibits phagocytic functions and induces apoptosis of macrophages (Gueirard et al., 1998; Khelef et al., 1993) as well as impairs chemotaxis and superoxide production in neutrophils (Friedman et al., 1987). The adenylate cyclase activity of CyaA was shown to account for these effects. However, later it was evidenced in experiments with murine macrophages J774A.1, that an increase of cAMP levels is not enough to account for the cytotoxicity but that also a permeabilization by the pore-forming activity of CyaA contributes to the cell death (Basler et al., 2006; Hewlett et al., 2006). Furthermore, we observed in our laboratory that CyaA causes massive actin cytoskeleton rearrangements in macrophages manifested by an intensive membrane ruffling which is accompanied by an inhibition of macropinocytic uptake and complement-mediated phagocytosis (Kamanova et al., submitted). Similarly, DCs are invaded by CyaA toxin (see 4.2) (Bagley et al., 2002) suggesting that CyaA not only incapacitates phagocytic and antimicrobial functions of innate immune cells but also interferes with adaptive immune functions of DCs in order to enable long-lasting infection of the bacteria. The molecular mechanisms of CyaA interaction with DCs is currently being examined in our laboratory. Specifically, if this toxin might interfere with Ag processing and presenting capacity of DCs will be investigated in my Master’s thesis.
4.2 Interaction of CyaA with DCs

CyaA was shown to interact with murine bone marrow-derived DCs (BMDCs) as well as human peripheral blood monocytes, precursors of DCs, thereby modulating the function of these cells in innate and adaptive immunity (Bagley et al., 2002; Ross et al., 2004). It was shown that CyaA inhibited LPS-induced upregulation of CD40, ICAM-1 and CD86, but not MHC class II molecules and even enhanced expression of CD80 (Boyd et al., 2005; Ross et al., 2004) in mouse bone marrow-derived DCs (BMDCs) treated with LPS or CpG, indicating that CyaA possesses a differential effects on maturation of DCs in the presence of maturation stimuli. Similarly, infection of BMDCs with wild type *B. bronchiseptica* significantly decreased the surface expression of CD40, but not MHCII, CD86 and CD80 when compared to CyaA-deficient bacteria (Skinner et al., 2004). No effect of CyaA on MHC II, CD86 and CD80 expression was also observed when DCs were infected with wild type or CyaA-deficient *B. pertussis* (Shumilla et al., 2004; Spensieri et al., 2006).

CyaA also affects LPS-induced secretion of cytokines. It inhibits TNF-α, IL-12p70 and MIP-1α (CCL3) production in DCs treated with LPS (Bagley et al., 2002; Ross et al., 2004; Skinner et al., 2004). The production of IL-12p70 was also inhibited when DCs were infected with wild type *B. pertussis* (Spensieri et al., 2006) as ΔcyaA mutants of *B. pertussis* released statistically significant higher amount of this cytokine which pronounced Th1 polarization. Furthermore, this group found that CyaA-mediated cAMP intoxication leads to the inhibition of IRF-1 and IRF-8 transcription factors by regulating the expression of IL-12p35 subunit which forms together with IL-12p40 mature active IL-12p70. CyaA also reduces the expression of IL-23 by affecting both p40 and p19 subunits gene expression (Spensieri et al., 2006). Ross et al. (2004) found that CyaA significantly augmented IL-6 and IL-10 production in LPS-treated DCs (Ross et al., 2004). Shumilla et al. (2004) working with ΔcyaA mutants of *B. pertussis* found equivalent levels of IL-10 production in infected DCs compared with wild type bacteria as well as Spensieri et al. (2006) indicating that other pathogenic factors of *B. pertussis* may also induce IL-10 production in DCs (Shumilla et al., 2004; Spensieri et al., 2006).

Mitogen activated protein kinases (MAPK) like p38 and ERK 1/2, and nuclear factor kappa B (NF-κB) pathways are involved in the regulation of DC’s maturation and cytokine production induced by TLR signalling (Rescigno et al., 1998; Sato et al., 1999). Data on the regulation of NF-κB and MAPK by CyaA are limited. It was found that CyaA inhibited the p38 MAPK in BMDCs infected with wild-type *B. bronchiseptica* but not by CyaA-deficient bacteria and had no effect on activation of ERK 1/2 (Skinner et al., 2004). On the other hand, Hickey et al.
(2008) showed, that CyaA alone induced ERK 1/2 phosphorylation and had no effect on activation of p38 in BMDCs. However, in combination with LPS, CyaA enhanced phosphorylation of p38 in these cells (Hickey et al., 2008). It was also this group which found that the treatment by CyaA alone led to a significant IkBα degradation at later time-points compared with LPS controls indicating that CyaA does not inhibit NF-κB translocation into nucleus. However, addition of CyaA did not affect IkBα degradation induced by LPS.

Interestingly, it was shown that cAMP generated by CyaA can also activate human monocyte derived DCs (MDDCs) to mature as determined by an increased expression of CD80, CD83, CD86 and HLA-DR and enhanced mixed lymphocyte reaction which suggests that CyaA might exert adjuvant properties (Bagley et al., 2002). CyaA was also shown to act as an adjuvant for antibody production in vivo. Hormozi et al. (1999) immunized mice with enzymatically active or inactive CyaA with or without ovalbumin (OVA) as the test Ag. The anti-OVA response was enhanced 3-4 fold in mice vaccinated with OVA and active CyaA compared to the mice receiving OVA alone. There was no significant enhancement of anti-OVA response when inactive CyaA and OVA were administered (Hormozi et al., 1999). Moreover, Ross et al., (2004) found significantly higher levels of keyhole limpet hemocyanin (KLH)-specific IgG1 antibodies in the serum of mice immunized with KLH and CyaA compared with mice that received Ag alone (Ross et al., 2004).

Recombinant enzymatically active as well as inactive CyaA was found to be efficient in delivering epitopes of various viral and bacterial proteins into the cell for presentation on MHC class I and II molecules for parallel induction of both T helper and T cytotoxic responses in vivo (Fayolle et al., 1996; Loucka et al., 2002). Moreover, mice immunized with CyaA carrying a CD8+ T cell epitope of OVA developed strong epitope-specific CTL responses, which killed tumber cell expressing this Ag showing its potential for immunotherapy (Fayolle et al., 1999).

### 4.3 CyaA-generated cAMP signalling modulates DCs

Cyclic adenosine 3′,5′-monophosphate (cAMP) is a prototypical second messenger, which modulates various physiological processes in all domains and kingdoms of life. In mammalian cells there are three known types of cAMP effector proteins: protein kinase A (PKA), guanine nucleotide-exchange protein directly activated by cAMP (EPAC) and cyclic nucleotide gated ion channels (Kamenetsky et al., 2006). Recently, Phosphodiesterase type 10 has been identified as a possible fourth target of cAMP signalling (Gross-Langenhoff et al., 2006). In mammalian cells cAMP is degraded by phosphodiesterases.
As it has been already mentioned, CyaA influences the expression of costimulatory molecules as well as the level of cytokines in LPS-treated DCs. To find out whether this is caused by an increased level of intracellular cAMP, the pharmacological cAMP-elevating agents were used in mimic the effects of CyaA. Bagley et al. (2002) showed that incubation of MDDCs with a permeable cAMP analogue di-butryryl cAMP (db-cAMP) or forskolin, which activates membrane adenylate cyclases in cells, increased the expression of CD80, CD83, CD86 and HLA-DR on these cells similarly to the purified CyaA (Bagley et al., 2002). This proved that cAMP can directly influence the expression of these molecules in DCs. An effect of cAMP signalling on IL-12p70 production in DCs was also observed. When MDDCs were infected with ΔcyA mutant of B. pertussis alone, no inhibition of IL-12p70 was detected, but this could be reversed by an addition of db-cAMP to the infected cells (Spensieri et al., 2006). In other study the modulation of DCs by CyaA-generated cAMP was shown using enzymatically inactive CyaA which did not inhibit CD40 and ICAM-1 expression and TNF-α, IL-12p70 and CCL3 production CpG-stimulated BMDCs (Boyd et al., 2005). All of these data established very well that CyaA modulates maturation and cytokine production in DCs by its ability to generate cAMP.

Direct effects of cAMP elevating agents on DCs have also been studied as it is known that an increase of intracellular cAMP in immune cells generally leads to a suppression of their inflammatory function. Whereas inhibitory effects of cAMP signalling on LPS-induced TNF-α and IL-12p70 and enhancement of IL-10 production are well established in the literature (Bagley et al., 2002; Galgani et al., 2004; Kambayashi et al., 2001), differential effects on DC’s maturation have been observed in dependence of a cAMP-elevating agent. In BMDCs cAMP analogue 8-Br-cAMP, lipid mediator prostaglandin E2 (PGE2) or inhibitor of phosphodiesterases IBMX inhibited LPS-induced up-regulation of MHC class I, MHC class II and CD40 expression and even slightly increased the expression of CD80 and CD86 (Kambayashi et al., 2001). In contrast, the addition of 8-Br-cAMP to MDDCs did not affect CD86, CD83 and HLA-DR up-regulation but inhibited the increase of CD54 expression induced by LPS (Galgani et al., 2004) suggesting that not only chemical compound but also the type and origin of DCs might determine the final outcome of cAMP signalling on DC’s maturation. This might be also relevant to cAMP signalling generated by CyaA toxin.

4.4 CyaA, cAMP and antigen processing and presentation

While maturation and cytokine production in LPS-stimulated DCs have been well documented, surprisingly there is not much known about CyaA influence on Ag presentation in DCs. Boschwitz at al. (1997) observed, that human monocytes were not able to stimulate
CD4⁺ T cell proliferation to tetanus toxoid when infected with wild type *B. pertussis* in contrast to cells infected with ΔcyaA mutant (Boschwitz et al., 1997) suggesting that CyaA impairs T cell activation. The elevation of intracellular cAMP in BMDCs by cAMP-elevating agents (8-Br-cAMP, PGE2, IBMX) caused significantly lower proliferation of peptide-specific CD4⁺ T cells compared to LPS controls (Kambayashi et al., 2001). On the other hand, it was shown in *in vivo* experiments with subcutaneously immunized mice, that CyaA promotes the induction of Th2 and T regulatory type 1 (Tr1) cells specific for the co-administered antigen, and that Th1-type T cells may also be generated, but at lower frequency (Ross et al., 2004).

In our laboratory, we found that CyaA-treated BMDCs significantly decreased MHC class I presentation of OVA protein as well as to a lesser extend OVA_{257-264} peptide compared to BMDCs treated with enzymatically inactive CyaA as measured by IL-2 production of CD8⁺ T cell line B3Z. Moreover, we observed that CyaA, in the presence of LPS, also decreased MHC class II presentation of OVA protein, but not OVA_{258-277} peptide in BMDCs in comparison to detoxified CyaA-treated cells as measured by IL-2 production of CD4⁺ T cell line MF2.2D9. Similar effects were observed using db-cAMP to artificially increase cAMP levels in BMDCs (Adkins et al., unpublished). As we observed that CyaA does not affect the capacity of DCs to present OVA peptide, but diminishes the ability to present OVA protein it is conceivable that CyaA might affect Ag uptake and/or processing in DCs in order to impair CD4⁺ T cell adaptive responses. Moreover, recent findings in our laboratory showed that CyaA inhibits the fluid phase uptake of dextran-FITC in J774A.1 macrophages (Kamanova et al., submitted). In experiments with db-cAMP the same results were obtained suggesting that elevation of intracellular cAMP concentration by CyaA is important for the inhibition of macropinocytosis by this toxin (Kamanová et al., submitted). Recently, we have also observed the inhibition of macropinocytosis in DCs (Adkins et al., unpublished) suggesting that this might be together with a decrease in MHC class I molecule expression (Adkins et al., unpublished) one of the mechanisms for the inhibition of CD8⁺ T cell responses. Nevertheless, there might be other factors e.g. decreased CD40, CD54 expression or IL-10 production responsible for the impairment of T cell adaptive responses (Fig. 3).

However, nothing is known so far about the effects of CyaA on Ag uptake by other endocytic pathways such as receptor-mediated endocytosis as well as on Ag processing in DCs. There are studies showing that cAMP signalling might affect these processes in immune cells. Cholera toxin (CT) of *V. cholerae* and heat labile enterotoxin (LT) of enteropathogenic *E. coli* modulate MHC class II Ag processing in macrophages (Matousek et al., 1998; Matousek et al., 1996). CT and LT are enterotoxins that compose of A and B subunits. The enzymatically
active A subunit of these toxins mediates ADP-ribosylation of the Gsα subunit of G proteins which results in the activation of adenylate cyclases and the accumulation of intracellular cAMP. It was shown that CT, and to a lesser extent LT, have a negative effect on an intracellular processing of hen egg lysosome (HEL) protein in murine macrophages (Matousek et al., 1998). CT or LT treatment of macrophages enhanced presentation of soluble HEL_{48-61} peptide to 3A9 CD4+ T cell hybridoma. Furthermore, CT and LT had no effect on Ag uptake (Matousek et al., 1996). Similarly, experiments with CT-treated murine B lymphoma cells showed remarkable inhibition of their ability to present OVA, but not OVA_{323-339} peptide to CD4+ T-cells (Tanaka et al., 1999). The authors found that the increase of intracellular cAMP level elevated pH in acidic intracellular compartments which led to reduced degradation of the Ag. Another study also showed that increased cAMP levels by 8-Br-cAMP reduced phagosome acidification in macrophages (Kalamidas et al., 2006).

Taken all these facts together, we can suppose that CyaA by the enhancement of intracellular cAMP may have effects on Ag processing in order to incapacitate T cell adaptive responses. Whether CyaA interferes with Ag processing capacity of DCs represents the main objective of my future work.

4.5 Bacterial interference with antigen presentation

Many bacteria subvert Ag uptake, processing and/or presentation of APCs which represents an important strategy to prevent stimulation of T cell adaptive immune responses in their host. Similarly to cholera toxin of *V. cholerae* and *E. coli* enterotoxin, toxin VacA from *Helicobacter pylori* was shown to interfere with proteolytic processing of tetanus toxin and toxoid and specifically inhibits the l-dependent pathway of Ag presentation in autologous antigen pulsed EBV-transformed B cells (Molinari et al., 1998). Intracellular bacteria *Chlamydia trachomatis* have evolved a specific mechanism for disrupting IFN-γ signalling pathways via degradation of upstream stimulatory factor (USF-1), thereby inhibiting MHC class II expression in human cells (fibroblasts, mammary epithelium cell line, HeLa) (Zhong et al., 1999). It was shown that IFN-γ-induced cell surface expression of HLA-DR molecules was markedly attenuated by viable *Mycobacterium tuberculosis* in THP1, human monocyte cell line, as well as in primary monocytes (Hmama et al., 1998). *M. tuberculosis* was also found to inhibit MHC class II expression and Ag processing in macrophages which was caused by a newly identified 19-kDa lipoprotein (Noss et al., 2001). Cheminay at al. (2005) demonstrated that *Salmonella enterica* inhibited Ag presentation in DCs, which was dependent on proteins encoded on *Salmonella* pathogenicity island 2 (SPI2) and secreted by the type III secretion system (Cheminay et al., 2005).
Fig. 3 An overview of data on CyaA interaction with LPS-stimulated DCs known from the literature as well as obtained in our laboratory (Adkins et al., unpublished) (Black). Whether CyaA interferes with Ag processing in DCs is the main objective of my future work (Blue).

5. References


