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Mechanism of action of adenylate cyclase toxin on immune function of dendritic cells

Dissertation Thesis

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Statement of originality

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

Prague,

Signature

I would like to thank my supervisor prof. Peter Šebo for introducing me to science and for giving me the opportunity to perform this work. I admire greatly his scientific enthusiasm and endless energy.

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Declaration of the co-authors:

I declare that Martina Švédová contributed (10 - 75 %) to the creation of the six specialized publications, which are part of this dissertation. She was involved in a significant way in performing experiments, their planning, the interpretation of results and writing of the publications.

Prague, prof. Ing. Peter Šebo, CSc.

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1. ABSTRACT

The adenylate cyclase toxin (CyaA) is a key virulence factor of the whooping cough agent *Bordetella pertussis*. CyaA primarily penetrates CR3-expressing myeloid phagocytes and subverts cellular signaling by a rapid conversion of ATP to cAMP. In parallel, CyaA can form cation-selective pores within cellular membrane, provoking massive potassium efflux from cell cytosol. An enzymatically inactive adenylate cyclase toxoid (CyaA-AC$^-$) has then been abundantly used as an efficient antigen delivery tool over the past 20 years.

This work focused mainly on the mechanism of action of CyaA toxin and of its toxoid on dendritic cells. We studied the potency of the CyaA toxoid to act as adjuvant, its penetration capacity and its potential use in delivery of influenza epitopes. We show that the pore-forming activity and the activation of MAP kinases JNK and p38 were crucial for the adjuvant effects of the CyaA-AC$^-$, which provokes maturation of dendritic cells (DC) independently of Toll-like receptor (TLR) or inflammasome signaling. Furthermore, such CyaA-AC$^-$-stimulated DC acquired the ability to induce CD8$^+$ and CD4$^+$ T cells responses, as was determined both *in vitro* and *in vivo*. We further showed that the first 371 amino acids are dispensable for the capacity of CyaA to deliver its AC domain with inserted epitopes into cytosol of DC, implicating that the role of the AC domain polypeptide in the process of translocation across the cytoplasmic membrane of cells is rather passive. Our CyaA toxoid construct with an inserted antigen from the HA2 subunit of the hemagglutinin of influenza A viruses then induced both humoral and cellular immune responses in mice without the need for any added adjuvant and the responses protected mice against challenge with both homologous and heterologous influenza A viruses.

We further examined the role of the CyaA toxin in virulence of *B. pertussis*. We analysed the modulatory effects of CyaA action on TLR-activated murine and human DC. CyaA enhanced TLR-induced dissolution of cell adhesive contacts and chemotactic migration of DC *in vitro*, while it decreased the capacity of DC to present protein antigens and induce proliferation of antigen-specific CD4$^+$ and CD8$^+$ T cells. Manipulation of mouse DC by CyaA *in vitro* was shown to depend solely on the cAMP signaling and not on the pore-forming activity of the toxin. We further demonstrated in the mouse respiratory challenge model that the pore-forming activity of CyaA was not required for bacterial colonization. It, however, provoked neutrophil infiltration and the pore-forming activity importantly contributed to the overall pathology of lungs infected by *B. pertussis*.

2. ABSTRAKT

Adenylátcyklázový toxin (CyaA) je klíčovým faktorem virulence bakterie *Bordetella pertusis*, která je původcem černého kašle. CyaA se váže na fagocyty, které exprimují na svém povrchu komplementový receptor 3 a poté katalyzuje rychlou přeměnu ATP na cAMP, které rozvrací buněčnou signalizaci. Zároveň CyaA tvoří uvnitř buněčné membrány kation-selektivní póry, které zapříčiňují únik draslíku z buněčného cytosolu. Enzymaticky neaktivní forma CyaA toxinu, adenylátcyklázový toxoid (CyaA-AC$^-$), se používá jako nástroj pro dopravu antigenů již 20 let.

Tato práce se zaměřila především na studium mechanismu působení CyaA toxoidu a toxinu. Zkoumali jsme adjuvantní účinky CyaA toxoidu, jeho kapacitu nořit se do buněčné membrány a možnost jeho využití pro dopravu epitopů z chřipkového viru. Ukázali jsme, že pórotvorná aktivita toxoidu a následná aktivace MAP kináz JNK a p38 jsou klíčové pro adjuvantní účinek toxoidu CyaA-AC$^-$ a způsobují maturaci dendritických buněk (DC), nezávislou na signalizaci TLR drah a inflamazómu. K stimulaci dendritických buněk dochází dokonce i *in vivo* a toxoidem aktivované DC jsou pak schopné navodit CD8$^+$ a CD4$^+$ T buněčné odpovědi *in vitro* a *in vivo*. Dále jsme ukázali, že prvních 371 aminokyselin je postradatelných pro schopnost CyaA dopravovat vložené epitopy do buněčného cytosolu, a že tudíž role AC domény při procesu penetrace toxinu do buňky je spíše pasivní. Konstrukt CyaA toxoidu s vloženým antigenem HA2 podjednotky hemaglutininu chřipkového viru A navodil látkovou i buněčnou imunitní odpověď v myších bez použití dalšího adjuvans a ochránil je proti infekci homologním i heterologním chřipkovým virem.

Také jsme zkoumali roli CyaA toxinu při infekci. CyaA manipuluje lidské a myší DC stimulované TLR tak, že dochází ke zvýšenému zániku buněčných adhezivních kontaktů, následnému zvýšení chemotaktické migrace a snížení schopnosti dendritických buněk předkládat bílkovinné antigeny a navodit tak proliferaci antigen-specifických CD4$^+$ a CD8$^+$ T buněk. Ukázali jsme, že CyaA manipulace myších DC *in vitro* je závislá výhradně na cAMP signalizaci, nikoli na pórotvorné aktivitě CyaA. Na myším modelu jsme dále prokázali, že pórotvorná aktivita CyaA není nutná pro bakteriální kolonizaci, avšak zvyšuje infiltraci neutrofilů a významně přispívá k plicní patologii během pertusové infekce.

3. ABBREVIATIONS

AC	adenylate cyclase
APC	professional antigen-presenting cell
ATP	adenosine triphosphate
BMDC	bone-marrow derived dendritic cells
CR3	complement receptor 3
CyaA (ACT)	adenylate cyclase toxin
CyaA-AC$^-$	adenylate cyclase toxoid
cAMP	cyclic adenosin monophosphate
DC	dendritic cell
AEC	airway epithelial cells
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
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PKA	protein kinase A
PFT	pore-forming toxin
PT	pertussis toxin
RTX	repeat-in-toxin
TLR	Toll-like receptors
Treg cells	T regulatory cells

4. INTRODUCTION

4.1 Immunity

Immunity is a basic defence mechanism of an organism. It preserves the organism's integrity by distinguishing harmful from harmless and thus protects the organism against a potential threat. This is the role of the **immunological recognition** which can eventually lead to an onset of effective immune response (**immune effector functions**). The **immune regulation** then supervises whether the immune response is appropriate. Additionally, it also guarantees the recognition and tolerance of its own cells. The protection against recurring infections is then ensured by the **immunological memory** (Murphy et al., 2008).

Mammalian immune system consists of an innate and of an adaptive immune system. The **innate immune response** plays a major role in the first days of infection and is rather unspecific, whereas the **adaptive immune response** begins later and is targeted specifically against an actual threat/pathogen. The adaptive immune system is responsible for generation of the immunological memory (Murphy et al., 2008).

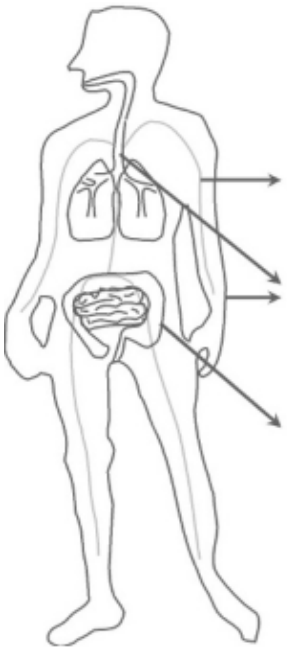
4.1.1 Airway immunity

Airway epithelial cells are able to specifically regulate the local immunity in the airway. They are the first barrier of the organism that meets various environmental compounds, be it potential allergens, pathogens or just harmless substances.

4.1.1.1 Graded immune response

It was proposed that there exists something like a "graded immune response" (Raz, 2007), which in fact means the ability of different organs to specifically regulate their own immune response. Hence, each organ can sense infectious agents in a different way. The reason for such a regulation of local immunity is the diverse degree of sterility in various body compartments, which correlates with the distribution of microbial flora within the organism. The human body can be divided into three different compartments (Fig. 1). The most sterile compartment is represented by blood, which is very sensitive to any danger. Any microbial

penetration can trigger a huge immune response in blood. On the other hand there are specialized compartments in the body which are in a permanent contact with bacteria. Thus, the immune response has to be supressed there. This situation refers to the gut. The airway is somewhere in between.



compartment	microbial contact	PRR sensitivity
blood	none	high every microbial contact indicates danger
airways, skin	frequent	regulated certain microbial load might be tolerated
gut	permanent	suppressed tolerance is dominating

Fig. 1: Organ specific regulation of immune response. Sensitivity of different body compartments to microbial contact. Microbial recognition within the innate immune system is achieved by the use of PRRs that are also found on epithelial cells. However, PRRs in general are not able to discriminate pathogenic from non-pathogenic microbes. Thus, pattern recognition must be modulated and regulated in an organ specific manner, especially within non-sterile compartments. It is proposed that PRR sensitivity is organ specifically-regulated. An important control variable is microbial load at a given anatomical site. PRR (pathogen recognition receptor) (Mayer & Dalpke, 2007).

Differential regulation of immune response is achieved through modulation of the sensitivity to pathogen associated molecular patterns (PAMPs). PAMPs are recognized mainly through toll-like receptors (TLRs). There are about ten TLRs which can be stimulated by certain bacterial components, i. e. LPS, flagellin etc. Upon stimulation, the TLRs are responsible for activation of signaling pathways that activate induction of immune response. There are several proposed mechanisms of regulation of TLR sensitivity such as restriction of apical TLR expression (TLRs are intracellular and only after an activation they are exposed on cell surface) and

absence of co-receptors needed for proper interaction or presence of inhibitory signaling molecules (Mayer & Dalpke, 2007).

4.1.1.2 Airway anatomy

Human airways are defined as the upper respiratory tract and the lower respiratory tract. The upper respiratory tract comprises the nasal passages, the paranasal sinuses and the pharynx. The lower respiratory tract begins at the larynx, continues to the trachea and divides into the two main bronchi until they eventually reach the alveoli (Fig. 2a).

The luminal surface of the airways is lined by a layer of respiratory epithelial cells - the so called pseudostratified columnar epithelium that can be seen in Fig. 2b. The pseudostratified columnar epithelial cells are a special type of cells that look like if there are more cell layers but in fact there is only one layer. It consists of ciliated and goblet cells which produce mucus and together they are responsible for the mechanism of the mucociliary clearance.

a) b)

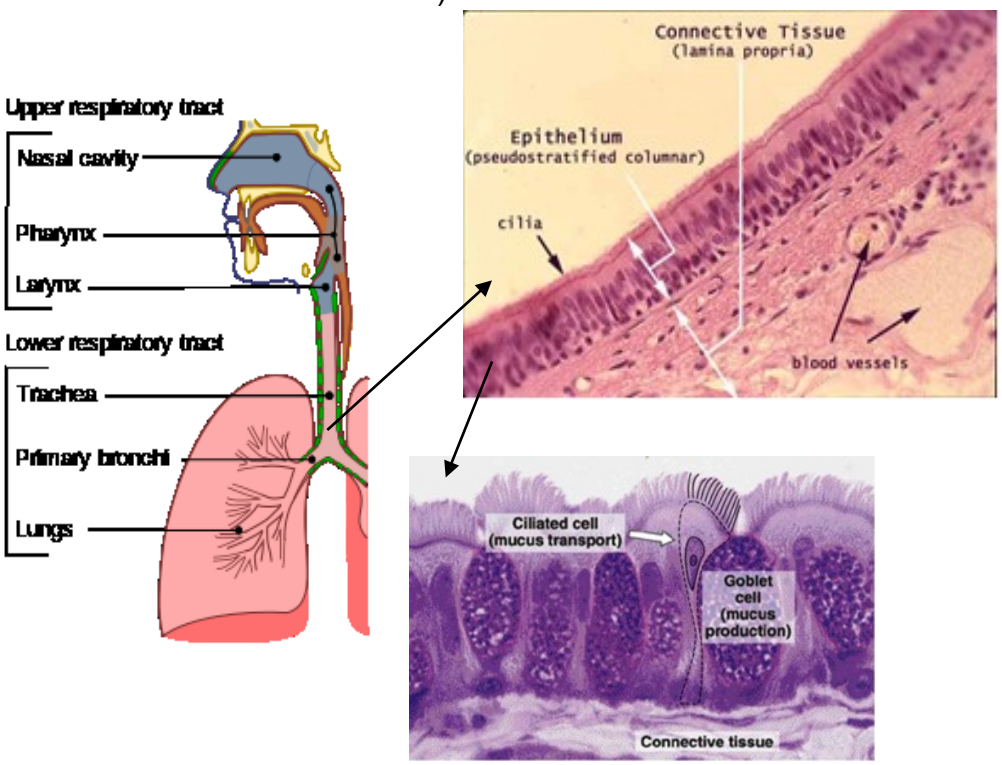


Fig. 2: Anatomical organization of the respiratory system. a) The respiratory tract is divided into the upper and lower airways. The upper airways includes the nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal cords. The lower airways includes the portion of the larynx below the vocal cords, trachea,

bronchi and bronchioles. The lungs include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli; from (https://en.wikipedia.org/wiki/Respiratory_tract). **b)** Airways are lined by the ciliated pseudostratified columnar epithelium with ciliated, goblet and basal cells. The ciliated cells are columnar epithelial cells with ciliary modification, goblet cells are columnar epithelial cells producing airway mucus and basal cells are small cuboidal cells endowed with the capacity to differentiate into other cell types in case of epithelium injury; from (http://academic.pg.cc.md.us/~mhubley/a&p/205/labimages.htm; https://quizlet.com/1144313/dit-respiratory-day-13-flash-cards/).

4.1.1.3 Innate and adaptive immunity of the airways

The respiratory tract is continuously exposed to the inhaled air, which may contain potentially pathogenic microorganisms. Therefore the airway epithelial cells have several mechanisms of prevention of bacterial infections.

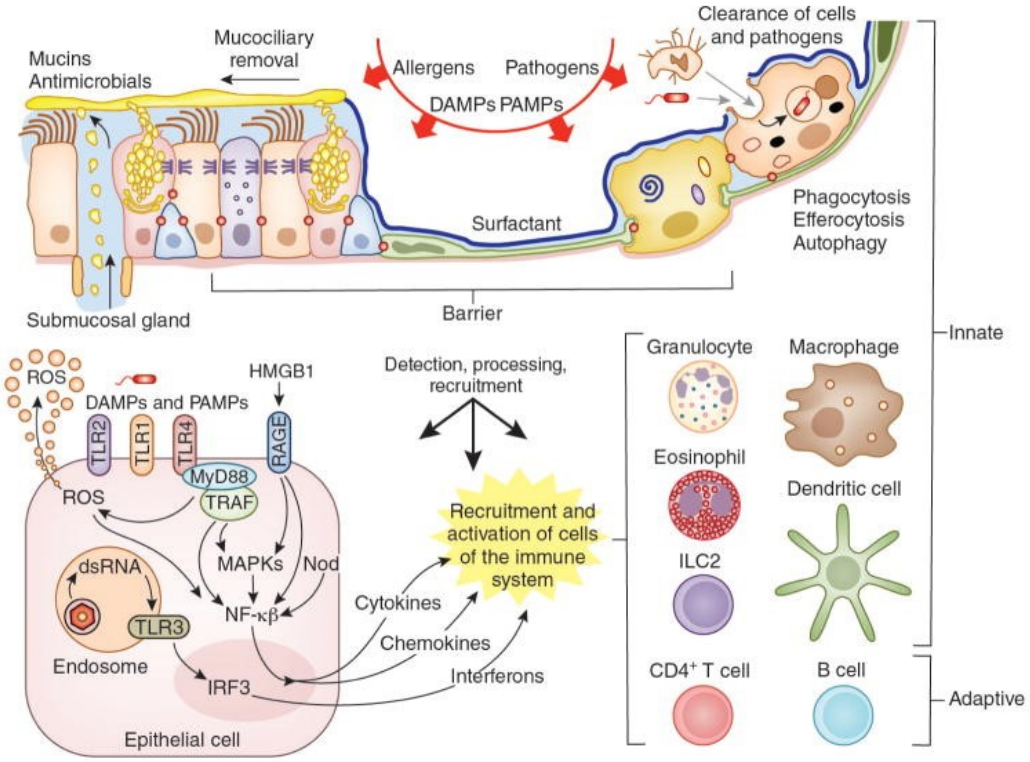
The first line of the innate immunity defence in the respiratory tract is the **physical removal of pathogens by mucociliary clearance and cough**. Mucociliary clearance describes the self-clearing mechanism of the airway. Cilia are the hair-shaped structures on the surface of respiratory epithelial cells (ciliated cells), which are surrounded by a thin fluid film of mucus secreted mainly by the goblet cells (non-ciliated cells). The cilia co-ordinately move the mucus in the direction towards the pharynx. Thereby the mucus, including some foreign particles or microorganisms, is transported to the mouth, where it can be either swallowed or coughed off (Diamond et al., 2000).

Moreover, **a broad spectrum of agents** with antibacterial, antifungal or antiviral activities such as lysozyme, lactoferrin, antimicrobial peptides (defensins, cathelicidins, collectins, pentraxins, LL-37), secretory leukocyte protease inhibitor (SLPI), serum amyloid A (SAA), nitric oxide, reactive oxygen species or prostaglandins are present in the mucus. These antimicrobial products are secreted both constitutively and inducibly by the epithelial cells and act through different mechanisms depending on their character: enzymes, permeabilizing antimicrobial peptides, opsonins, protease inhibitors, small toxic molecules, or binding/neutralizing macromolecules. The production of these substances by the airway epithelial cells is induced upon stimulation of their PRRs (Diamond et al., 2000; Kato & Schleimer, 2007).

In the case of infection, **phagocytic cells are recruited to the site of infection** due to chemoattractants produced by airway epithelium (Diamond et al., 2000; Kato & Schleimer, 2007). Moreover, epithelial cells have the ability to up-regulate the expression of surface molecule like ICAM-1, which acts as a ligand for the integrin CD11a/CD18 present on neutrophils, monocytes, lymphocytes and eosinophils, promoting thus interaction and transmigration (Diamond et al., 2000). In order to keep a balanced immune response, the airway epithelial cells produce also molecules like the cytokines IL-10 and TGF-ß, prostaglandin PGE2 and the soluble cytokine receptor antagonist sTNFR1, which have rather anti-inflammatory effects and thus significantly modify the final shape of the immune response (Kato & Schleimer, 2007).

However, the airway epithelial cells can also modulate the adaptive immune response. It was reported that they can modulate the phenotype of matured DC by interacting with them and thus altering their T cell stimulatory and response-polarizing capacity, which results in the manipulation of T cells response (Schleimer et al., 2007). Additionally, the airway epithelial cells can also directly modulate the effector T cell functions by production or expression of molecules that interferes with T cell activities (Kato & Schleimer, 2007).

a)



b)

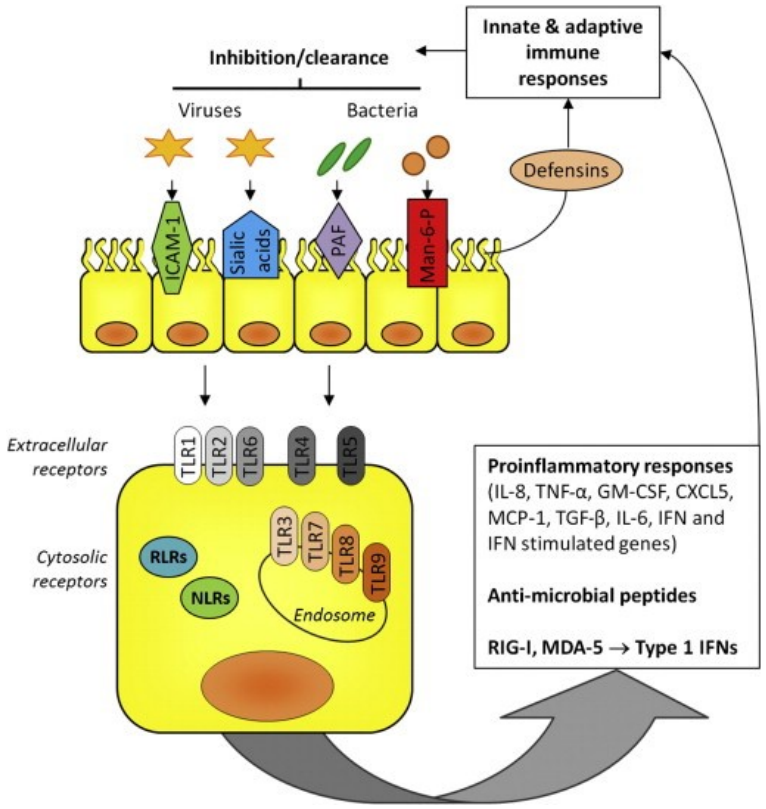


Fig. 3: The immune responses of airway epithelial cells. a) Signaling via PAMPs and DAMPs in respiratory epithelial cells and the downstream host defence responses. PAMPs derived from commensal microbes or respiratory pathogens and DAMPs generated

from cell stress and/or death, within both the conducting airways and alveoli, are recognized via membrane-associated or cytosolic PRRs expressed in respiratory epithelial cells. The binding of ligands to these receptors results in the activation of epithelial cell–intrinsic signaling pathways (via MAPK, IRFs, reactive oxygen species (ROS) and NF-κB) and subsequent production of cytokines, chemokines and antimicrobial proteins that recruit and activate cells of the innate and adaptive immune systems and regulate the barrier function. These same recognition pathways in epithelial cells can stimulate autophagy, phagocytosis and the clearance of necrotic cells and of pathogens and thus further influence the local inflammatory responses. Adopted from (Whitsett & Alenghat, 2015). **b) Cellular responses of the airway epithelium to infection.** Viruses and bacteria bind to cellular receptors such as intercellular adhesion molecule 1 (ICAM-1) and sialic acid residues, and platelet-activating factor (PAF) and mannose-6-phosphate receptors, respectively. This enables the pathogens to internalize and/or replicate in airway epithelial cells. This induces the production of innate immune defensins and the stimulation of extracellular and intracellular innate immune receptors (TLRs, RLRs, NLRs). This leads to the generation of pro-inflammatory innate and adaptive immune responses and the production of interferons (IFNs). Adopted from (Hallstrand et al., 2014).

4.1.2 Dendritic cells

Dendritic cells (DC) were first found in the mouse spleen and characterized by Steinman and Cohn (Steinman & Cohn, 1973). DC are well known to be the key players of immunity, serving as a bridge between innate and adaptive immune responses. DC are not only the initiators and potent stimulators of the immune response, but importantly, also induce self-tolerance.

4.1.2.1 Maturation of dendritic cells

Immature DC stay as sentinels in the periphery tissues, capturing and processing antigens into the MHC peptide complexes. After sensing pathogen or other danger, they undergo a process of maturation (Fig. 4). This is a very complex process during which DC up-regulate their co-stimulatory molecules (CD80, CD86, CD40 and CD54), down-regulate antigen uptake, enhance antigen processing and presentation and start producing inflammatory cytokines (TNF-α, IL-1 and IL-12). Furthermore, the DC start expressing chemokine receptors (CCR7) which enable

them to migrate to the secondary lymphoid tissue towards the homing chemokines. Finally, all these transformations give DC the ability to prime naïve T lymphocytes (Banchereau & Steinman, 1998).

Upon activation, the DC travel to the lymphoid tissue, where they stimulate naïve T lymphocytes. In order to do this, DC increase expression of the surface receptor CCR7. This allows them to migrate towards homing chemokines CCL19 and CCL21, which attract DC to the lymph nodes (Alvarez et al., 2008). However, this is sometimes not sufficient, as it was shown that another stimulus, such as prostaglandin E (PGE2), is required for activation of the CCR7 receptor (Scandella et al., 2002; Scandella et al., 2004).

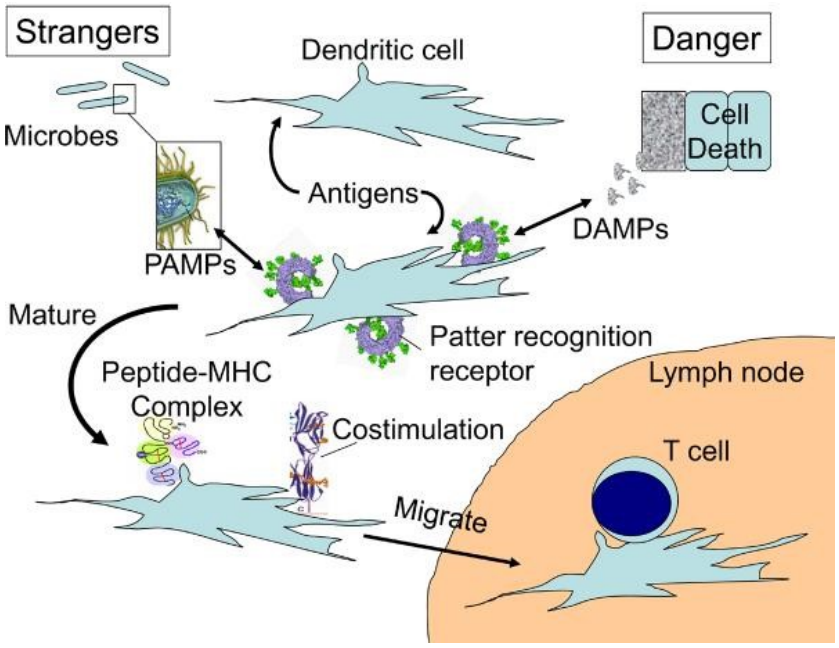


Fig. 4: Model of dendritic cell activation - DC as a bridge between innate and adaptive immunity. DC can be activated by various stranger or danger molecules, which result in DC maturation and migration to the lymph node. DC are present in all tissues, where they continuously sample antigens from the surrounding environment. There are two models of DC activation – the stranger and the danger model. According to the stranger model (Janeway), DC can recognize pathogens by pathogen-associated molecular patterns (PAMPs), which can activate the pattern-recognition receptors (PRRs). Whereas in the danger model (Matzinger), stressed or dying cells release danger signals – damage-associated molecular patterns (DAMPs), which then activate their corresponding receptors on DC. In both cases, DC are activated (enhancement of co-stimulatory molecules) and migrate to the lymphoid tissue, where they present antigens on MHC molecules and stimulate T cells. (TCR, T-cell receptor) (Kono & Rock, 2008)

The activation of DC can be induced by a large variety of stimuli: microbial products, endogenous (danger) signals and feedback signals from cells of the immune system (cytokines or direct contact with T cells) (Macagno et al., 2007). However, also other stimuli such as cAMP elevation (Garay et al., 2010), Ca^{2+} and K^{+} ion signaling (Matzner et al., 2008; Shumilina et al., 2011), contact sensitizers (Kagatani et al., 2010), or mechanical stress (Jiang et al., 2007) were shown to have the capacity to activate DC. Depending on the type of stimuli, the maturation state of DC can vary and lead to development of immunogenic DC or tolerogenic DC (Mellman, 2013). Subsequently, this specific state of DC maturation then shapes the resulting adaptive immune response. Different arrays of cytokines released by the differentially matured dendritic cells play an important role in determining T-cell outcomes. The production of IL-12 promotes the development of Th1 cells, whereas the production of IL-4 favours the development of Th2 cells and the production of IL-23 results in the development of Th17 cells. Transforming growth factor-β1 (TGFβ1) promotes the development of Treg cells, IL-10 induces Tr1 cells, and IL-6 also contributes to Th17 cell development (Romagnani, 2005).

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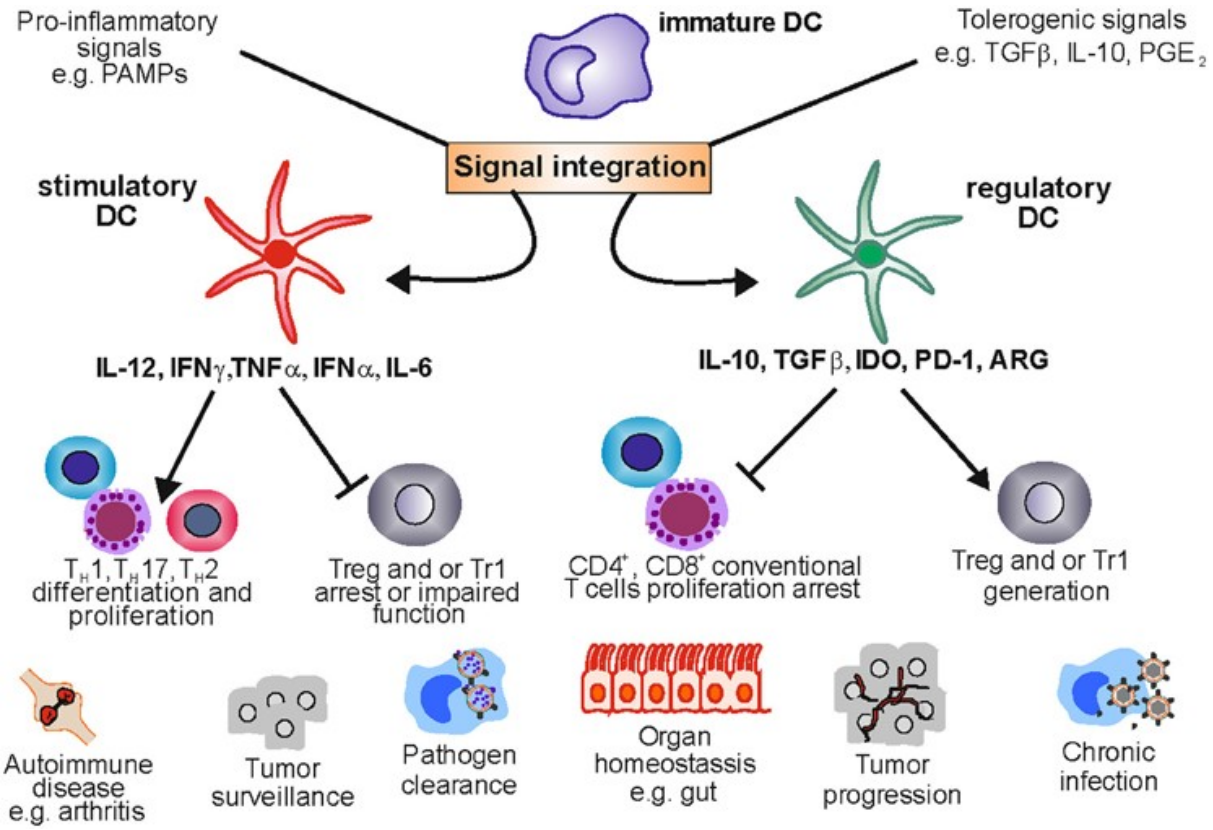

Fig. 5: Stimulatory and regulatory DC in health and disease. DC are a plastic lineage able to process and integrate signals from the microenvironment. Under pro-inflammatory conditions, stimulatory DC promote an effective immune response by stimulating T cell proliferation and shaping T cell responses toward Th1, Th2, or Th17 phenotypes. This crucial role allows the immune system to clear pathogens and keep transformed cells in check. Nevertheless, uncontrolled DC activation can lead to tolerance ablation, fostering the development of autoimmune diseases like rheumatoid arthritis. Under a tolerogenic environment, DC acquire regulatory functions, suppressing T cell activation and proliferation and providing signals that enable Treg and Tr1 differentiation and expansion. This function maintains tolerance in organs to a variety of harmless antigens, like the gut. However, regulatory DC function can be exploited by tumors and pathogens leading to tumor progression and chronic infection. Adopted from (Schmidt et al., 2012).

Subsets of DC

Both murine and human DC can be divided according to their phenotype and function to two main categories: conventional DC (plus inflammatory DC derived from circulating monocytes) and plasmacytoid DC. The conventional DC are located in peripheral tissues (skin and mucosal surfaces) where they take up antigens, upon stimulation migrate to afferent lymph nodes and prime naïve T lymphocytes. On the other hand, the plasmacytoid DC accumulate mainly in the blood and the lymphoid tissue and their task is to respond to bacteria and viruses by Type I interferon production. In Fig. 6 is a scheme of four major subsets and functional specialization of DC which can be found in mice and humans.

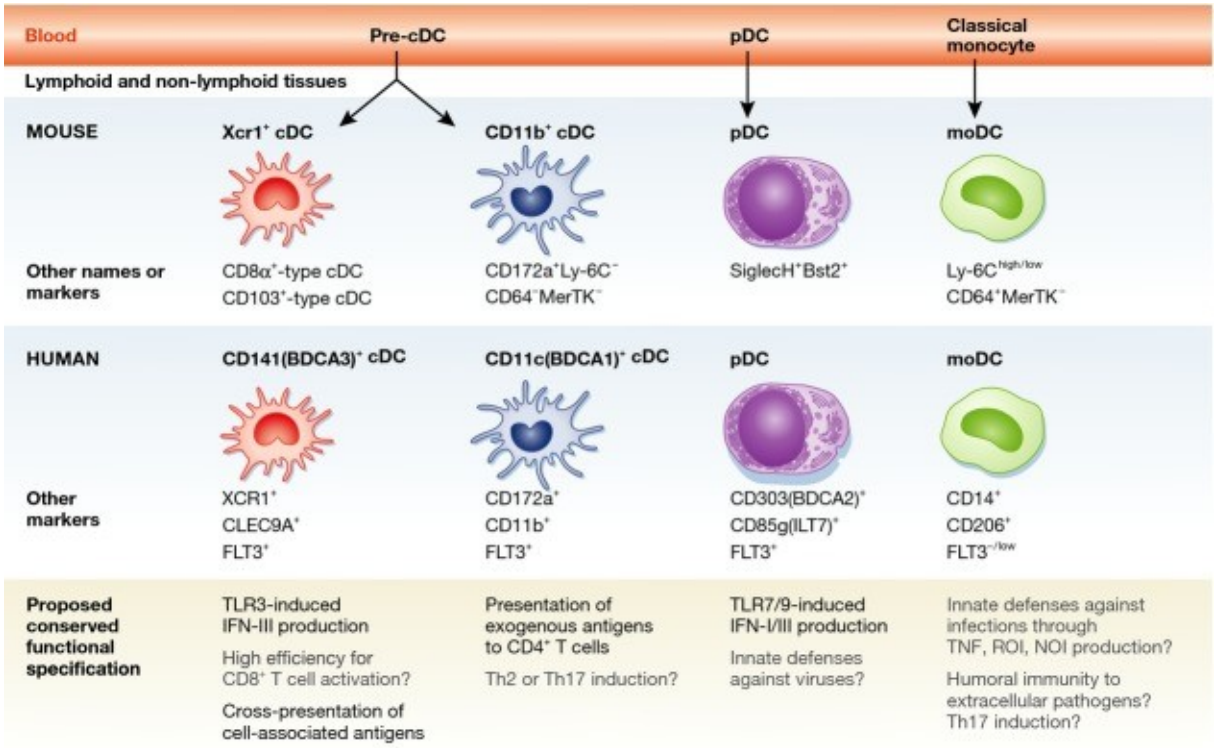

Fig. 6: Major subsets of DC in mouse and human. Human and mouse DC subsets can be aligned into four major subsets irrespective of their location in secondary lymphoid tissues or in the parenchyma of non-lymphoid organs. The precursors that are found in the blood and give rise to the four major DC subsets are shown. Alternative markers or names used to identify those subsets are also indicated, as well as the proposed conserved functional specialization of these subsets (Dalod et al., 2014).

4.1.3 Vaccination

Vaccination is considered as one of the greatest public health achievements of the twentieth century and it has contributed to a dramatic decline in mortality from infectious diseases. In the past, vaccines have been developed empirically, without or with only minimal understanding of immunological mechanisms that act in vaccine efficacy. Currently, we are trying to design vaccines rationally, using our improved immunological knowledge.

There are two types of vaccines. Live attenuated vaccines comprise weakened forms of the pathogens (example vaccines: chicken pox, rubella, measles, mumps). The immunity induced by these types of vaccines is similar to post-infectious immunity and thus it is usually quite effective and conferring long-lasting protection. The second type are subunit vaccines, consisting of only a specific part of

the pathogen (example vaccines: pertussis, diphtheria, tetanus, hepatitis B) (Pulendran & Ahmed, 2011).

4.1.3.1 Role and mechanism of various adjuvants

The acellular vaccines usually contain some adjuvant, a specific substance, which has the ability to potentiate (augment) the induced immune response. However, another effect of the adjuvant is that it also shapes the type of the immune response, often towards a different type of immune response than would occur during the natural course of infection.

Until now, only few adjuvants have been licensed for general use in humans. They are listed in the Table 1, together with their mechanism of action and type of the immune response they activate.

Table 1: Immune activation by adjuvants (Coffman et al., 2010; Pulendran & Ahmed, 2011)

Adjuvant	**Mechanism of action**	**Type of immune response**
Alum (aluminium salt)	Caspase-1 and NLRP3 inflammasome activation in DCs	Th2, Ab (+Th1 in humans)
AS04 (a combination adjuvant composed of monophosphoryl lipid A (a TLR4 ligand) adsorbed to alum)	TLR4 and inflammasome activation	Th1, Ab
MF59 and AS03 (squalene-in-water emulsion)	Unknown; probably enhanced uptake by APCs; tissue inflammation	Th1+Th2, Ab
Some of possible new adjuvants		
Flagellin-protein fusions	Activates TLR5 and inflammasome components	Th1 and Th2
TLR7 and TLR8 ligands	TLR7 ligands	Th1, Ab
CpG DNA	TLR9 ligand	Th1, Ab

Despite the extensive use of these adjuvants, little is known about their exact mechanism of action or their potential toxic effects. Further studies need to be conducted to find specific adjuvants that induce desirable immune responses and ideally create a lifelong memory.

4.2 Bacterial toxins

Bacterial toxins are important virulence factors produced by bacteria. They have the ability to promote infection directly by damaging host tissues or by manipulating host immune responses. Various bacterial toxins have different mechanisms of toxicity. Pore-forming toxins form pores within cellular membrane, disrupt ion homeostasis and eventually can cause the lysis of a cell. Other toxins manipulate important cellular signaling pathways, for example by elevating cAMP, a key second messenger molecule. Yet other toxins block protein synthesis and thus trigger host cell apoptosis.

Many bacterial toxins then have an interesting potential as useful tools for research. Even more important, toxins can be valuably used in the field of medicine (Adkins et al., 2012).

4.2.1 Pore-forming toxins

One of the most important groups of bacterial toxins are the pore-forming toxins (PFTs). This group comprises approximately 25 % of all known bacterial protein toxins. Although they are quite diverse proteins and the group is rather heterogenic, PFTs share similar mechanisms of the action. According to their name, they use an evolutionary ancient, but still very effective strategy: by compromising the permeability barrier of host cells membranes, these toxins significantly interfere with ion homeostasis of host cells (Linhartova et al., 2010). Nevertheless, hosts have developed efficient strategies to overcome this kind of bacterial attack, eliciting a robust transcriptional response. So we can speak about the general host cell response to PFTs (Bischofberger et al., 2012; Huffman et al., 2004; Kao et al., 2011; Los et al., 2013).

4.2.1.1 Host cell response to PFTs

The disruption of membrane integrity causes osmotic stress. Especially critical is the role of disruption of potassium homeostasis by excessive K^+ efflux and intracellular K^+ depletion, which can lead to apoptosis (Yu, 2003). Not surprisingly, the toxicity of PFTs is dose-dependent. At high concentrations of PFTs, the cells

cannot stand the high pore-forming activity within their plasma membrane and die quickly, whereas for low sublytic PFT concentrations, host cells have developed efficient strategies to counteract pore-formation. The key mechanism consists in the activation of mitogen-activated protein kinases (MAPKs) (Cancino-Rodezno et al., 2010; Porta et al., 2011; Kao et al., 2011). MAPK family is a group of highly conserved proline-directed, protein-serine/threonine kinases, which are activated by stress and control host systems involved in cell survival, proliferation and adaptation (Krishna & Narang, 2008). While ERK1/2 is considered to be the extracellular signal-regulated kinase, JNK and p38 are mainly stress-activated protein kinases. The p38 and JNK pathways play an important role in immunity (Cargnello & Roux, 2011; Dong et al., 2002). Interestingly, it was shown that loss of cellular potassium specifically triggers p38 phosphorylation (Kloft et al., 2009). This is now considered to be the conserved host response in nematodes, insects and mammals (Porta et al., 2011).

Yet another supporting data from genomic studies (microarray analysis), performed on the nematode *Caenorhabditis elegans,* revealed that the Cry5B toxin from *Bacillus thuringiensis* up-regulates p38 and JNK mRNA levels (or to be exact, their equivalent) (Huffman et al., 2004). Moreover, the elimination of either of these MAPK pathways resulted in hypersensitivity to low chronic dose or high brief dose of this toxin (Huffman et al., 2004). Even though the Cry toxin targets nematodes and insects (de Maagd et al., 2003), similar behaviour was observed also on mammalian baby hamster kidney cells treated with aerolysin (Huffman et al., 2004). This might reflect a general form of host strategy of coping with PFTs. Although the importance of p38 activation during the defence against PFTs was already shown earlier (Huffman et al., 2004; Husmann et al., 2006; Bischof et al., 2008; Cancino-Rodezno et al., 2010), Kao recently pointed out that it is dominantly the JNK pathway that regulates the transcriptionally-induced defences. The main actor in this play appears to be the downstream regulator AP-1, an ancient transcription factor, which is conserved from worms to humans (Kao et al., 2011).

Many of these cellular responses to PFTs include the immune signaling. MAPK p38 activates the IRE-1 pathway that influences the immune response via NF-κB activity (Xu et al., 2005). Furthermore, the IRE-1 pathway participates in phospholipid biogenesis that helps membrane restoration. It was reported that mRNA for the major neutrophil chemokine IL-8 (KC) accumulated upon p38 activation mediated by PFT(s) like vaginolysin (VLY) and (PLY) (Gelber et al., 2008; Ratner et

al., 2006), while anthrax lethal toxin (LeTx) down-regulates expression of IL-8 (Chow et al., 2010). Furthermore, streptolysin O (SLO) is able to induce production of several inflammatory mediators such as TNF-α, IL-13, IL-4, IL-6, MCP-1 and GM-CSF (Stassen et al., 2003).

The schematic representation of selected response pathways to PFT attack are depicted in Fig. 7. It is noteworthy that calcium and potassium act as the major regulators of cellular response to PFTs. As a consequence of drop in potassium level, the cell might respond by autophagy, arrest of global translation, formation of lipid droplets, activation of MAPK kinases, histone H3 dephosphorylation and activation of caspases 1 and 2, respectively. On the other hand, the rise in cytosolic calcium concentration can lead to rapid restoration of membrane integrity, plasma membrane fusion of secretory vesicles, activation of calpain and inhibition of mitochondrial function (Bischofberger et al., 2012).

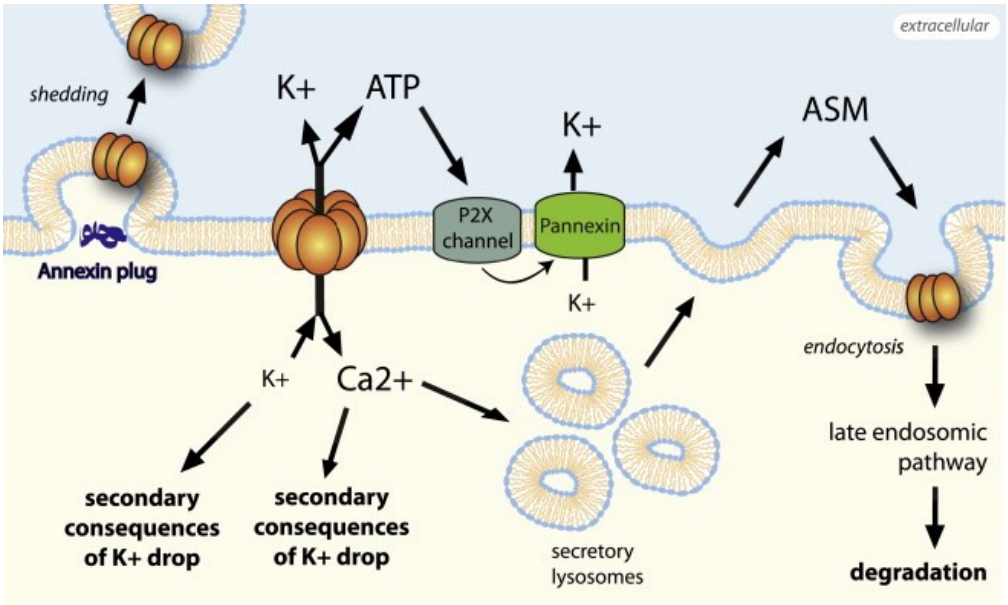

Fig. 7: Schematic representation of selected response pathways to PFT action. The described pathways have been reported but do not necessarily apply to all PFTs. Calcium entry through the pore may lead to the calcium dependent formation of membrane blebs that may initially be isolated from the rest of the cell by an annexin plug. If membrane repair fails in the blebs, the membrane domain can be shed off the cell. Calcium entry may also trigger the fusion of secretory lysosomes that contain acid sphingomyelinase (ASM). Upon secretion, ASM cleaves the head groups of sphingomyelin and phosphatidyl choline, which in turns triggers endocytosis, leading to uptake of the PFTs. Efflux of ATP through the pore may

lead to the activation of P2X channels, which in turn leads to opening of the pannexin channels, allowing massive potassium efflux. Adopted from (Bischofberger et al., 2012).

4.2.2 Bacterial toxins in antigen delivery and immunotherapy

Bacterial toxins have been extensively studied for almost a century now. Their mechanism of action helped to elucidate many processes within the cell. Due to the better understanding of their behavior, toxins can now be wisely manipulated to act for beneficially purpose. Over the last 30 years, bacterial toxins and their mutated variants have been exploited as vectors for antigen delivery, as adjuvants, or in immunotherapy of various infectious, malignant and autoimmune diseases.

Several toxins were reported to have the capacity to deliver antigens into host cells for antigenic presentation and stimulation of specific T cell responses. The examples comprise *Bordetella pertussis* adenylate cyclase toxin, *Bacillus anthracis* lethal and edema toxins, *Shigella dysenteriae* shiga toxin, *Escherichia coli* shiga-like toxin and the effector proteins of the type III secretion system of *Yersinia* or *Salmonella.*

Some bacterial toxins have the potential to act as adjuvants and stimulate mucosal and/or systemic immune responses (e.g. *Vibrio cholera* cholera toxin, *Escherichia coli* heat-labile enterotoxin, *Bordetella pertussis* pertussis toxin or the Cry1A protein of *Bacillus thuringiensis*). Other toxins were shown to be effective in immunotherapy such as the *Corynebacterium diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A.

Below are listed only a few examples of bacterial toxins exhibiting an interesting potential of practical use:

Anthrax toxins of *Bacillus anthracis*: *B. anthracis* is the causative agent of anthrax. There are three components of anthrax toxins: protective antigen (PA), lethal factor (LF) and edema factor (EF). PA plus LF in combination form the lethal toxin which causes death of experimental animals (H. Smith & Keppie, 1954), whereas PA plus EF form the edema toxin, which increases cellular cAMP levels and can cause skin edema (Leppla, 1982; Stanley & Smith, 1963). PA binds to its receptors, the tumor endothelium marker 8 and the capillary morphogenesis protein

2, both of which are expressed also on the surface of antigen presenting cells (Bradley et al., 2001; Scobie et al., 2003). Then, PA is cleaved, oligomerizes into heptameric pores, binds LF or EF and the complex is endocytosed (Klimpel et al., 1992; Gupta et al., 2001; Kumar et al., 2001). With the help of PA, the LF and EF subunits are able to translocate from the endosomes into cell cytosol (Milne et al., 1993; Guidi-Rontani et al., 2000). While EF is an adenylate cyclase (Leppla, 1982), the toxicity of LF lies in its ability to cleave MAPK kinases (Duesbery et al., 1998).

Modified LF and modified LF/EF with PA were, indeed, exploited as a potential antigen delivery system. This was shown to successfully generate T cell responses in many settings (Chandra et al., 2007; Adkins et al., 2012). It is noteworthy that mice immunized with LF/EF - PA constructs bearing antigens from *L. monocytogenes,* or LCMV, were protected against lethal challenge (Ballard et al., 1996; Doling et al., 1999). The enzymatically active ET was shown to act as an adjuvant for co-administered antigens (Duverger et al., 2006; Maldonado-Arocho et al., 2009). In addition, PA with LF were exploited for cancer immunotherapy and PA-L1 + LF construct was shown to induce 90 % tumor growth inhibition and 30 % complete regressions of the V600E BRAF melanoma cell line C32 xenografts in mice (Liu et al., 2000).

Cholera toxin of *Vibrio Cholerae* and heat-labile enterotoxin of *Escherichia coli*: *V. cholera* and enterotoxigenic strains of *E. coli* are responsible of cholera and traveller's watery diarrhoea, respectively. Both toxins have similar AB_5 structure, bind to gangliosides receptors on cell surface (Holmgren et al., 2003) and are delivered to the cell cytosol via the retrograde transport pathway. Upon translocation of the A subunit, they ADP-ribosylate G proteins provoking increase of cytosolic cAMP (Gill & Woolkalis, 1991).

The AB_5 cholera toxin (Ctx) and heat-labile enterotoxin (Etx) are known to be potent mucosal adjuvants (Elson & Ealding, 1984; Clements et al., 1988). However, not only toxins, but importantly also their nontoxic mutants, or only their B subunit oligomers, were shown to exert adjuvant properties (Del Giudice & Rappuoli, 1999; Salmond et al., 2002). Moreover, these toxins were shown to deliver antigens as well. Both of the modified toxins were tested in clinical trials as part of the cholera or enterotoxigenic *E. coli* vaccines (Silva et al., 2008; Lapa et al., 2008). In addition to that, both toxins could modulate the immune response. While these toxins rather

stimulate the immune response, their nontoxic variants can induce tolerance (Salmond et al., 2002; Sun et al., 2010). Toxoids were reported to be very efficient in moderation of immune responses in a variety of autoimmune diseases or other immunopathological conditions, such as diabetes or Behcet's disease (Bergerot et al., 1997; Stanford et al., 2004).

Pertussis toxin of *Bordetella pertussis*: Pertussis toxin (PT) is another important virulence factor of *B. pertussis*. PT belongs to the superfamily of AB_5 toxins. It binds to sialic acid on cell surface and uses the retrograde transport pathway through Golgi apparatus to the endoplasmic reticulum, where the A subunit translocates to the cytosol (Plaut & Carbonetti, 2008). A subunit then ADP-ribosylates small heterotrimeric G proteins, which are responsible for regulation of the endogenous adenylate cyclases, resulting in elevated cAMP levels within host cells (West et al., 1985). PT has several inhibiting effects on the immune system during the course of *B. pertussis* infection (Carbonetti et al., 2003; Carbonetti, 2010).

PT was shown to act as an adjuvant (Roberts et al., 1995; Samore & Siber, 1996). Moreover, mutated PT without ADP-ribosylating activity still exhibited adjuvant properties, as was shown in numerous works (Roberts et al., 1995; Ryan et al., 1998; Ausiello et al., 2002; Wang et al., 2006). The safety and immunogenicity of its non-toxic form (PT9K/129G) was confirmed in clinical trials (Podda et al., 1990; Del Giudice & Rappuoli, 1999). Besides, PT or its PT-B oligomer was reported to have the capacity to inhibit some viral infections, as was shown in the mouse model of HIV infection (Lapenta et al., 2005).

Shiga toxin of *Shigella dysenteriae* and shiga-like toxin of *Escherichia coli*: These AB_5 toxins (Stx and Stx1) are involved in dysentery, haemorrhagic colitis or hemolytic uremic syndrome. They belong to the AB_5 family of protein toxins, with enzymatically active A part and B_5 part oligomer responsible for the binding to surface receptors on cells, which is the globotriose-ceramide in this case. The toxin is transported via retrograde pathway and after the translocation into cell cytosol, the A part inhibits protein synthesis and causes cell death (Sandvig & van Deurs, 1996; Tesh, 2010).

The nontoxic B_5 oligomer part was shown to both deliver antigens and to act as an adjuvant (Lee et al., 1998; Noakes et al., 1999; Choi et al., 2005). Although no maturation of BMDC after incubation with StxB was observed (Vingert et al., 2006), the nasal administration or *in vitro* incubation with Stx1B induced the expression of maturation markers on DC (Ohmura et al., 2005). Shiga toxin possesses also a potential to be an effective antiviral agent (al-Jaufy et al., 1994; Ferens et al., 2006). Interestingly, it can be used to specifically target cancer cells (El Alaoui et al., 2007) or be injected in some tumors as it was reported to inhibit tumor growth in mice (Farkas-Himsley et al., 1995; Ishitoya et al., 2004) .

Listeriolysin O of *Listeria monocytogenes*: Listeriosis can be dangerous for immunocompromised individuals and pregnant woman. Listeriolysin O (LLO) belongs to the family of cholesterol-dependent cytolysins. The toxin can be activated in phagosomes and thus enables bacteria to egress into cell cytosol (Portnoy et al., 1992; Gedde et al., 2000).

LLO has been used for antigen delivery into MHC class I and II pathways. Moreover, a variety of viral and tumor antigens fused with LLO have been shown to generate antigen-specific $CD4^+$ and $CD8^+$ T cell responses and to induce anti-tumor immunity in mice (Dietrich et al., 2001). Besides, LLO has been expressed in a wide variety of attenuated live bacterial vaccines to help them reach the cytosol and consequently be processed by MHC class I presentation pathway for $CD8^+$ T cell stimulation (Dietrich et al., 1998; Dietrich et al., 2003). In addition to that, LLO was shown to possess an adjuvant capacity and it induces DC maturation and production of pro-inflammatory cytokines (Yamamoto et al., 2006). An LLO based construct (*Lm*-LLO-E7) was shown to be safe in immunotherapy of cervical carcinoma (Maciag et al., 2009). Furthermore, live-attenuated Listeria vaccines ANZ-100 and CRS-207 (expressing tumor-associated antigen mesothelin), tested in phase I clinical study in patients with liver metastases and mesothelin-expressing cancers, were safe and resulted in immune activation (Le et al., 2012). Recently, it was shown that the CRS-207 vaccine with GVAX (GMCSF-secreting allogeneic pancreatic tumor cells) prime extended survival of patients with pancreatic cancer while exhibiting minimal toxicity (Le et al., 2015).

Diphteria toxin of *Corynebacterium diphteriae*: Diphteria toxin (DT) is a very potent toxin which causes a serious respiratory distress. The toxin consists of three domains: an enzymatically active A domain, a translocation T domain and a receptor binding domain B. It uses surface receptor binding, endocytosis, retrograde pathway and translocation from endosomes to deliver its A domain into the cell cytosol (Cabiaux et al., 1993). This results in ADP-ribosylation of elongation factor 2 (Bennett & Eisenberg, 1994) and consequently in apoptosis of host cells (Brinkmann et al., 1995).

Detoxified diphtheria toxin is used in diphtheria vaccines. Moreover, it has been used as a carrier in glycoconjugate vaccines to enhance the immunity response against several bacterial pathogens, which was important particularly for a significant reduction of *Haemophilus type b* and *Streptococcus pneumoniae* infection in children (Hausdorff et al., 2009). Besides, DT was shown to deliver various antigens as well (Stenmark et al., 1991). Interestingly, the toxin (or its part or fused mutants) was used in many other fields, exploiting its unique properties: for eradication of HIV-infected cells (Brdar et al., 2002; Alfano et al., 2005), in the treatment of autoimmune diseases (Woodworth & Nichols, 1993; Gottlieb et al., 1995), for cancer gene therapy (Maxwell et al., 1986; Lidor et al., 1997), or for the immunotherapy of cancer (Ohana et al., 2002; Mizrahi et al., 2009).

Exotoxin A of *Pseudomonas aeruginosa*: *P. aeruginosa* is an opportunistic bacterial pathogen which can cause infections in immunocompromised individuals and particularly in cystic fibrosis patients. Often, it is also responsible for nosocomial infections. The structure of Exotoxin A (PE) toxin is similar to the DT toxin, with three different domains being responsible for binding, translocation and enzymatic activity/toxicity, respectively (Allured et al., 1986). Similarly, also the route of toxin penetration into cell cytosol and mechanism of cytotoxicity are alike, yielding apoptosis of the host cell (Smith et al., 2006; Koopmann et al., 2000; Iglewski et al., 1977; Jenkins et al., 2004).

PE in its non-toxic form was used as a vector for antigen delivery. To mention some of the studies: the mutant PE variant with OVA polyepitope induced $CD8^+$ T cell protective immunity against lethal OVA-expressing tumor challenge in mice (Becerra et al., 2003). DNA vaccines or recombinant protein vaccines comprising part of PE fused to E7 (antigen from HPV16) generated strong specific $CD8^+$ T cell-

mediated immunity, protective against E7-expressing tumors (Hung et al., 2001; Liao et al., 2005). PE immunotoxins were shown to act also as anti-viral agents, but unfortunately, they failed in Phase I clinical trials (Ramachandran et al., 1994). However, some improved forms of PE immunotoxin were prepared and shown to be effective in depletion of HIV-infected cells (Berger et al., 1998). Additionally, PE was reported to be a strong mucosal and also systemic immunogen (Chen et al., 1999; Mrsny et al., 2002; Challa et al., 2007). In the contrary to DT, the clinical trials did not confirm any advantage of using PE in glycoconjugate vaccines (Pier, 2007).

DT and PE immunotoxins: Immunotoxins are molecules consisting of a protein toxin and a ligand (usually an antibody, growth factor or cytokine). On the surface of the target cell, the ligand binds to a tumor-associated antigen and delivers the toxin into cell cytosol, which eventually leads to cell death. Several immunotoxins have already been tested in clinical Phase I trials. A PE immunotoxin (HA22) is currently ongoing further trials (phase III) and a DT immunotoxin (DAB(389)IL-2) is already used for treatment of cutaneous T cell lymphoma (Wolf & Elsasser-Beile, 2009; Kreitman, 2009).

Some other toxins have other interesting capacities. Botulinum neurotoxin of *Clostridium botulinum* was shown to be useful for treatment of neurological disorders or in cosmetic industry and interestingly, cytotoxic necrotizing factor 1 of *Escherichia coli*, besides its adjuvanticity, was shown to improve learning and memory (Fabbri et al., 2008).

4.3 Bordetella pertussis and infection

4.3.1 Bordetella

Bordetellae are small, Gram-negative aerobic coccobacilli. The genus comprises nine species: *B. pertussis, B. bronchiseptica, B. parapertussis*, *B. parapertussis*$_{ov}$ (ovine-adapted), B*. avium*, *B. hinzii*, *B. holmesii*, *B. trematum*, and *B. petrii*. However, only some of the species are associated with respiratory infections in mammals. These are mainly due to *B. pertussis, B. bronchiseptica* and *B. parapertussis*. *B. pertussis* is a strictly human pathogen and it is a causative agent of

whooping cough (pertussis). *B. parapertussis* causes a milder pertussis-like infection, while *B. bronchiseptica* can infect a wide range of mammalian species, occasionally causing respiratory infections also in humans (Mattoo & Cherry, 2005). *Bordetellae* have a close phylogenetic relationship and a similar mechanism of pathogenesis. The diseases are transmitted by infected droplets from other hosts and bacteria then adhere to cilia of respiratory mucosa. The virulence of these three *Bordetella* strains is controlled by a two-component BvgAS system that regulates the expression of virulence factors in response to changes in extracellular signals from the surrounding environment (Mattoo & Cherry, 2005; Preston et al, 2004).

4.3.2 Bordetella pertussis infection

Bordetella pertussis was first isolated by Bordet and Gengou in 1906, but whooping cough (pertussis) has been known since the sixteenth century as a highly contagious, acute respiratory illness, which could be particularly severe in infants and children (Mattoo & Cherry, 2005). While pertussis is often associated with infants and young children, also adults may be infected. More importantly, adult often serve as carriers of the pathogen (Birkebaek et al., 1999; Rocha et al., 2015).

Despite extensive immunization world-wide, pertussis remains the least controlled vaccine-preventable infectious disease. Currently, there are about 16 million pertussis cases reported annually worldwide and about 195,000 children deaths occur per year (http://www.cdc.gov/pertussis/countries.html).

Bordetella pertussis infection can last 6 to 12 weeks, or even longer. It is a long-lasting illness, as is also evident from the Chinese term for whooping cough disease, which can be translated “the cough of hundred days“. It has three stages: catarrhal, paroxysmal and convalescent. After the incubation period, usually of 7 to 10 days, patients develop symptoms similar to the common cold, such as heavily runny nose, sneezing and mild cough. However, the body temperature remains rather normal. One reason why pertussis is so dangerous for infants is that it is the sole major infectious disease that is not accompanied by fever, until the critical pneumonia develops. Therefore, pertussis or whooping cough are typically

diagnosed too late, at a stage where application of antibiotics to the diseased infant has little effect.

The catarrhal stage continues for one or two weeks, with the cough getting gradually worse. The classical symptom of the paroxysmal stage is a paroxysmal cough characterized by an inspiratory “whoop” as air rushes into the lungs against a narrowed glottis at the end of a cough. This paroxysmal episodes occur in a group and may be followed by an exhaustion. Often, the paroxysms are associated with tenacious mucus and vomiting. Interestingly, between paroxysms the patients seem normal, without any respiratory problems (Mattoo & Cherry, 2005). Other systemic manifestations of pertussis could include: lymphocytosis, dehydration and malnutrition caused by posttusive vomiting, dysregulated insulin secretion, alteration in neurological function and paroxysmal cough occurring after the infection (Hewlett et al., 2014). The paroxysmal stage lasts commonly for 2 to 8 weeks with the slow transition into the convalescent stage, which continues for another 1 to 2 weeks or often even longer. The paroxysms in this stage are less frequent and milder (Mattoo & Cherry, 2005). However, pertussis may be more serious in infants causing necrotizing bronchiolitis, intraalveolar haemorrhage and fibrinous edema. Severe cases then manifest by extreme lymphocytosis, pulmonary hypertension and respiratory failure (Paddock et al, 2008).

The pertussis disease starts by inhalation of aerosol droplets containing *B. pertussis*. Subsequently, bacteria colonize the mucosa of the upper respiratory tract, where they produce a variety of virulence factors (see further Fig. 9) (Carbonetti, 2007; Mattoo & Cherry, 2005). It was shown that bacterial tracheal cytotoxin (TCT) and LOS synergize in the induction of IL-1α and NO production in hamster trachea epithelial cells (Flak et al., 2000). Further, the use of organ culture model of hamster trachea revealed that bacteria adhere to cilia, cause mucin hypersecretion and epithelial damage resulting eventually in decreased mucociliary clearance (Soane et al., 2000). In line with that is the fact, that the human bronchial epithelial cell line BEAS-2B was shown to up-regulate mRNA expression of genes encoding IL-6, IL-8, chemokine MCP-1 and two genes inducing mucin secretion (MUC2 and MUC5AC) after interaction with *B. pertussis* (Belcher et al., 2000). However, *B. pertussis* not only adheres to, but may also invade human tracheal epithelial cells (Bassinet et al., 2000). It was thought that the bacteria within the cells are efficiently killed (Bassinet

et al., 2000; Gueirard et al., 2005). However, Lamberti et al. recently showed that a significant portion of *B. pertussis* is able to survive within the human epithelial cell line A549 for several days (Lamberti et al., 2013).

The cause of pertussis persistence could be that many *B. pertussis* virulence factors exert important immunomodulatory actions and thus suppress the proper immune response. This leads probably to bacterial persistence and severity of disease. In Fig. 8 is a summary of immunomodulatory effects of some of the virulence factors of *B. pertussis*. Both the innate as well as the adaptive immunity is affected. Interestingly, we still have no idea why pertussis makes people cough. Cherry even proposed there could be some yet undiscovered “cough toxin” (Cherry, 2013).

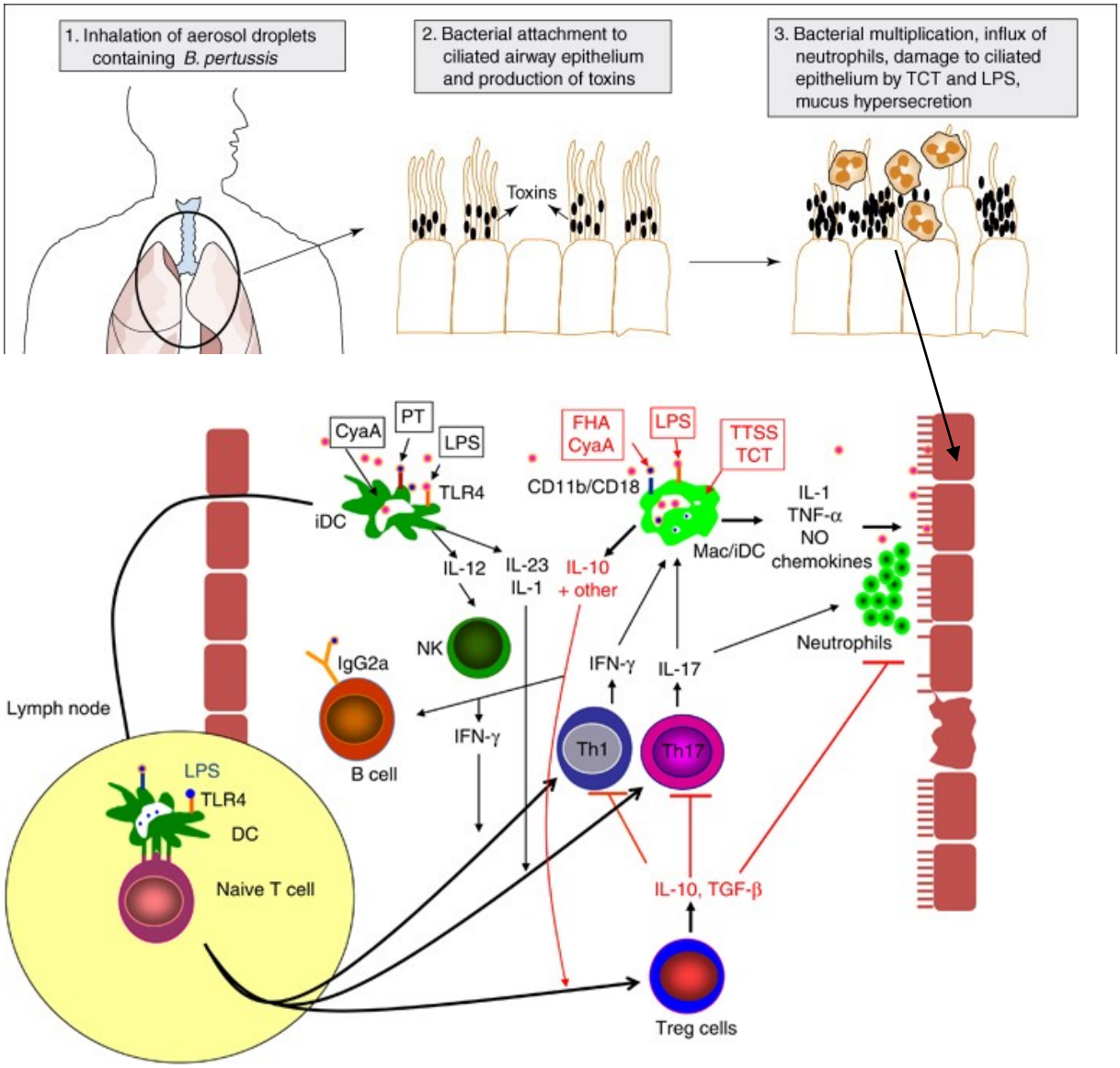

Fig. 8: The onset of *Bordetella pertussis* infection and immunity to *B. pertussis* in naïve host (evidence from the murine respiratory challenge model). When *B. pertussis* enters the respiratory tract, the bacteria bind to ciliated epithelial cells, but are also recognized and taken up by resident or infiltrating cells of the innate immune system, such as macrophages (Mac) and immature dendritic cells (iDC). DC process and present bacterial antigens to T cells. The production of interleukin (IL)-12 by innate cells results in polarization of the T-cell response to the T helper type 1 (Th1) subtype, and IL-1β and IL-23 drive the differentiation of Th17 cells. However, in early infection the local T-cell responses are suppressed by innate cells that secrete IL-10 in response to filamentous hemagglutinin (FHA), adenylate cyclase toxin (ACT), or type III secretion system (TTSS) and by the consequent regulatory T (Treg) cells. Nitric oxide (NO) and the pro-inflammatory cytokines, IL-1 and tumor necrosis factor (TNF-α) are induced by bacterial toxins, especially lipopolysaccharide (LPS), tracheal cytotoxin (TCT), ACT, and pertussis toxin (PT), and as well as contributing to bacterial elimination, also mediate local lung pathology and are responsible for many of the systemic and neurological consequences of the infection. Interferon (IFN-γ) secreted early in infection by DC and natural killer (NK) cells, and later in infection by Th1 cells, stimulates recruitment and activation of macrophages and neutrophils and provides help for B cells to secrete opsonizing and complement-fixing antibody (immunoglobulin (Ig) G2a in the mouse). IL-17 secreted by Th17 cells promotes macrophage inflammatory protein 2 production and neutrophil recruitment and activation. Opsonized or non-opsonized bacteria are taken up by neutrophils and macrophages, which are killed by NO or reactive oxygen intermediates. Black arrows represent inflammatory/effector immune responses and red arrows represent anti-inflammatory and immunosuppressive responses. TGF, transforming-growth factor; Adopted from (Carbonetti, 2007; Higgs et al., 2012).

Immunity to *B. pertussis*

As depicted in Fig. 8, protective immunity to *B. pertussis* is quite complex. Innate immunity controls the infection with the help of dendritic cells, macrophages, neutrophils, natural killer cells and antimicrobial peptides. Evidence suggests that neutrophils and macrophages activated by cytokines secreted by NK and $CD4^+$ T cells are importantly involved in clearing *B. pertussis* infection (Higgs et al., 2012). Moreover, pertussis infection induces secretion of antibodies, which were shown to be involved in vaccine-induced adaptive immunity to *B. pertussis* in a mouse model (Mills et al., 1998). However, for successful *Bordetella* clearance, Th1 cells producing IFN-γ were shown to be essential (Ryan et al., 1997; Mascart et al., 2003; Mahon et al., 1997; Barbic et al., 1997). In addition, also Th17 cells were shown to help in

clearance of bacteria from the respiratory tract of infected mice (Dunne et al., 2010). Similarly, in baboons the natural infection results in very strong Th1 and Th17 responses (Warfel & Merkel, 2013; Warfel et al., 2014). This is in agreement with the fact that an effective immune response for mainly an extracellular pathogen *B. pertussis* will require the induction of a mixed Th1/Th17 response stimulating the production of opsonizing, toxin-neutralizing and mucosal antibodies and memory T cells, producing cytokines to recruit and activate professional phagocytes (Melvin et al., 2014). On the contrary, *B. pertussis* subverts host protective immune response in order to prolong the infection. This is facilitated mainly by induction of regulatory T cells (Coleman et al., 2012).

Also other studies supported the crucial role of Th1 cells for an effective host immune response against *B. pertussis* infection, as summarized by Higgs et al. (Higgs et al., 2012). This explains well the efficacy of previously used wP vaccines that promoted Th1 type of immunity. On the contrary, aP vaccines induce predominantly Th2 type responses (Ryan et al., 1998; Ausiello et al.,1997), suggesting an explanation for the increasing emergence of whooping cough among populations in the most developed countries that switched recently to aP vaccines.

Vaccination

Whole-cell pertussis vaccines (wP), introduced in the 1940s, were effective in decreasing both morbidity and mortality caused by the whooping cough disease. Because of their reactogenicity, however, the wP vaccines have been replaced by the safer but less efficient acellular pertussis vaccines (aP) in many developed countries since 1990s (Preston et al., 2002; Higgs et al., 2012) As a result, the incidence of pertussis has increased and many epidemic outbreaks have been reported since then in developed countries (Poland, 2012). The deficiencies of aP vaccines were recently also confirmed by experiments in baboons, showing that the aP vaccine protects only from pertussis symptoms, but not from colonization by the pathogen or transmission of the disease (Warfel & Merkel, 2013).

Melvin summarized strategies, mainly three possible ways, which are currently under investigation in order to make vaccination against pertussis work again: (i) the inclusion of additional antigens in aP vaccines, (ii) switching the adjuvant in aP vaccine, which would polarize immunity more toward Th1/Th17 responses (in contrast to Th2 response generated by now used alum adjuvant) and (iii) the

development of live, attenuated *B. pertussis* vaccines, which would be safer than the previously used wP vaccines (Melvin et al., 2014).

4.3.3 Virulence factors and manipulation of host immunity

Bordetella pertussis produces a wide spectrum of virulence factors. Filamentous hemagglutinin (FHA), fimbriae (FIM), pertractin (PRN) and tracheal colonization factor (Tcf) are responsible for the adhesion of the bacteria to ciliated epithelial cells. Other virulence factors are toxins, such as the adenylate cyclase toxin (CyaA), pertussis toxin (PT), dermonecrotic toxin (DNT), endotoxin or lipooligosaccharide (LOS) and tracheal cytotoxin (TCT). There are also molecules which act as complement resistance factors: *Bordetella* resistance to killing A (BrkA) and Vag8 proteins. The schematic representation of *Bordetella pertussis* virulence factors is shown in Fig. 9 and their immunomodulatory effects in Fig. 10.

Furthermore, *B. pertussis* expresses the **type 3 secretion system** (T3SS), which has the ability to inject effectors directly across the plasma membrane into the target cell. Five genes encoding proteins secreted by T3SS were found in *B. pertussis* genome: BopN, BopB, BopD, BopC and Bsp22 (Fennelly et al., 2008).

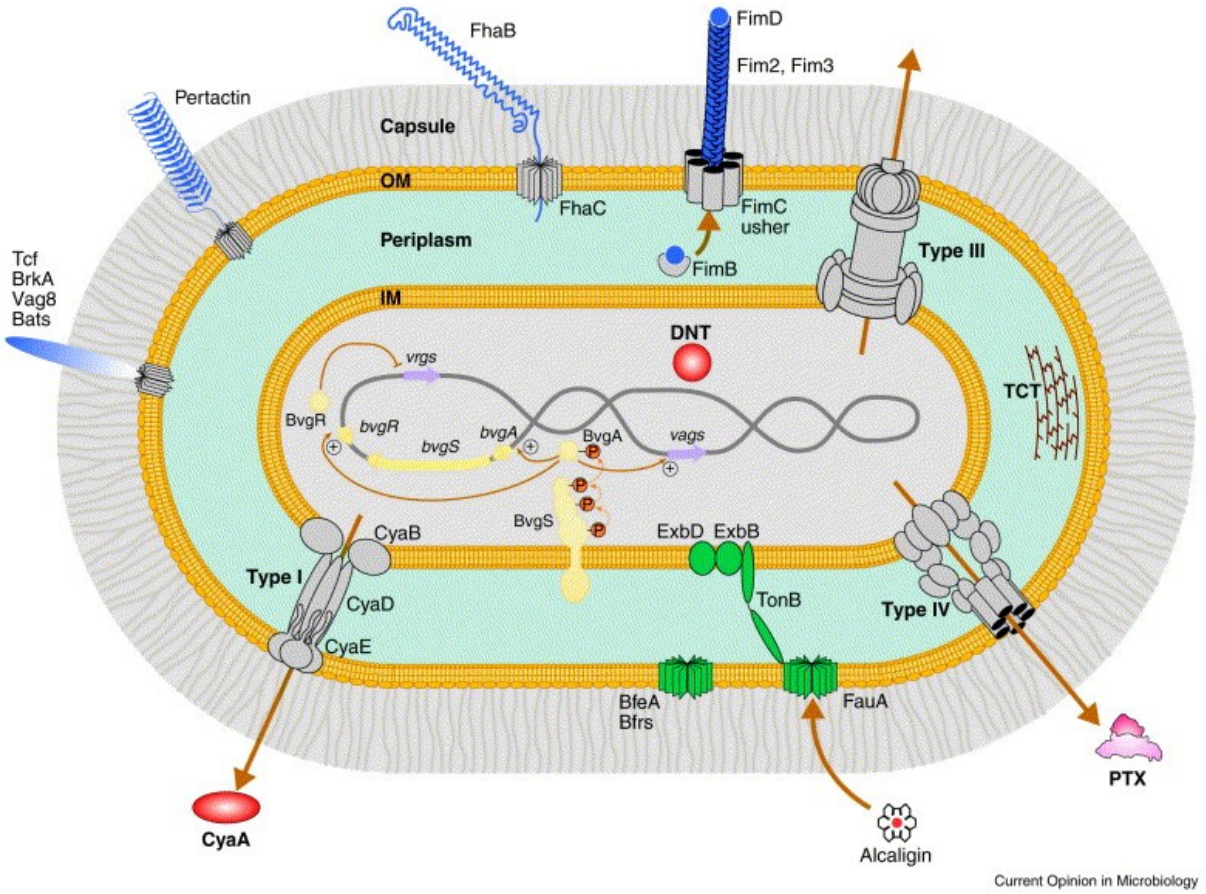

Fig. 9: *Bordetella petussis* virulence factors. *B. pertussis* is depicted as a Gram-negative organism with inner and outer membranes (IM and OM), a periplasm and a capsule. The adhesins Fim, FhaB, pertactin, Tcf, BrkA, Vag8 and Bats are shown in blue; the toxins PTX, CyaA and DNT are in red; the accessory 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. Adopted from (Locht et al., 2001).

Below is the description of the most important virulence factors of *B. pertussis* (except the CyaA toxin which will be dealt with in more details elsewhere):

Filamentous hemagglutinin, a rod-shaped protein, serves as a major adhesin *in vitro* and as an important colonization factor *in vivo* (Inatsuka et al., 2005). It was proposed to bind to the complement receptor 3 (CR3), the very late antigen V (VLA-5) and leukocyte response integrin/integrin associated protein (LRI/IAP) due to its interaction with its RGD (Arg-Gly-Asp) motif (Van Strijp et al., 1993). It was also

reported that FHA has the capacity to suppress immune response, however, recently Villarino et al., showed, that these observations were probably due to LPS contamination of FHA (Villarino et al., 2016). FHA is a component of the acellular pertussis vaccine.

Fimbriae (type1 pili) consist of two protein subunits Fim2 and Fim3, the expression of which can undergo phase variation (Willems et al., 1990). According to the studies on tracheal explants, fimbriae play a role in mediating adherence to ciliated respiratory epithelium (Edwards et al., 2005). Moreover, FIM were shown to be necessary for colonization of the lower respiratory tract in rodents (Geuijen et al., 1997; Mattoo et al., 2000). Recently, Scheller et al., confirmed in *in vivo* adherence assay using *B. bronchiseptica* and mice that FIM are, indeed, important adhesins, which mediate initial interaction with airway epithelial cells allowing then tight FHA binding. FIM and FHA then together mediate suppression of inflammation, leading to prolonged bacterial colonization (Scheller et al., 2015).

Pertractin belongs to the family of classical autotransporter of outer membrane proteins. While PRN was shown to participate in adhesion on ciliated rabbit tracheal explant cultures (Edwards et al., 2005), the importance of PRN was not confirmed in *in vivo* experiments in mice (Khelef et al., 1994). This is also reflected by the fact that recently *B. pertussis* strains lacking PRN have been isolated from pertussis patients (Bouchez et al., 2009; Pawloski et al., 2014). PRN is included in the aP vaccine and current evidence indicates the loss of PRN production in circulating *B. pertussis* strains due to aP vaccine pressure (Guiso, 2014; Bouchez et al., 2015; Safarchi et al., 2015).

Pertussis toxin is an exclusive virulence factor of *B. pertussis*. (Its structure and the mechanism of toxicity are described above). It contributes to pertussis pathogenesis, but it is not the only molecule responsible for pathogenicity as believed before. During infection, PT probably initially targets alveolar macrophages (Carbonetti, 2007). Many activities of PT were identified. *In vitro* inhibition of migration of neutrophils, monocytes and lymphocytes (Spangrude et al., 1984) was later confirmed in mouse models (Kirimanjeswara et al., 2005; Andreasen & Carbonetti, 2008). PT is also the cause the extreme lymphocytosis observed in patients (Mu et

al., 1994). As a key virulence factor and protective antigen (Bruss & Siber, 1999), PT is an important component of all aP vaccines (Carbonetti, 2015). Antibodies against PT prevent critical pulmonary pertussis in infants (Robbins et al., 2009).

Dermonecrotic toxin, a typical A-B toxin, consists of an N-terminal receptor-binding and a C-terminal enzymatic domain. It has a transglutaminase activity and activates small Rho family GTPases (Schmidt et al., 1999). It causes necrotic skin lesions after being injected subcutaneously (Cowell et al., 1979). Interestingly, DNT is not secreted from bacterial cells grown in culture and it would only be released upon bacterial cell lysis (Cowel et al., 1979). The role of DNT during *B. pertussis* infection remains unknown. It was shown that in the mouse model, the *B. pertussis* mutant not producing DNT was as virulent as the wild-type bacteria (Weiss & Goodwin, 1989).

Lipooligosaccharide is the *B. pertussis* form of LPS lacking the O-antigen (Caroff et al., 2001). LOS can stimulate TLR4, but it is less potent in that than LPS (Mann et al., 2005; Marr, 2010). It is important to mention that human and murine TLR4-MD2-CD14 complexes differ in their response to various forms of LPS. It seems that human response to LOS is stronger than in mice (Melvin et al., 2014). Only recently it was shown that the capacity of *B. pertussis* LOS to stimulate TLR4 signaling depends on GlcNac modification of lipid A (Maeshima et al., 2015).

Tracheal cytotoxin is the only known virulence factor of *B. pertusiss* that is not regulated by BvgAS. TCT is produced during cell wall remodeling, but unlike the most Gram-negative bacteria which reuse it, *B. pertussis* releases a large amount of this disaccharide-tetrapeptide monomer of peptidoglycan (Cookson et al., 1989). It was shown that TCT and LOS from *B. pertussis* synergize in the induction of pro-inflammatory cytokines (IL-1α, L-1β, IL-6 and TNF-α) and NO production, leading eventually to epithelial cell destruction in the model of hamster tracheal epithelial cells (Flak et al., 1999; Heiss et al., 1993). This is caused by the response of NOD1, a PPR sensitive to peptidoglycan (Magalhaes et al., 2005). However TCT is only poorly recognized by human NOD1 (Magalhaes et al., 2005), which raises a doubt whether TCT acts in pertussis pathogenesis any significantly.

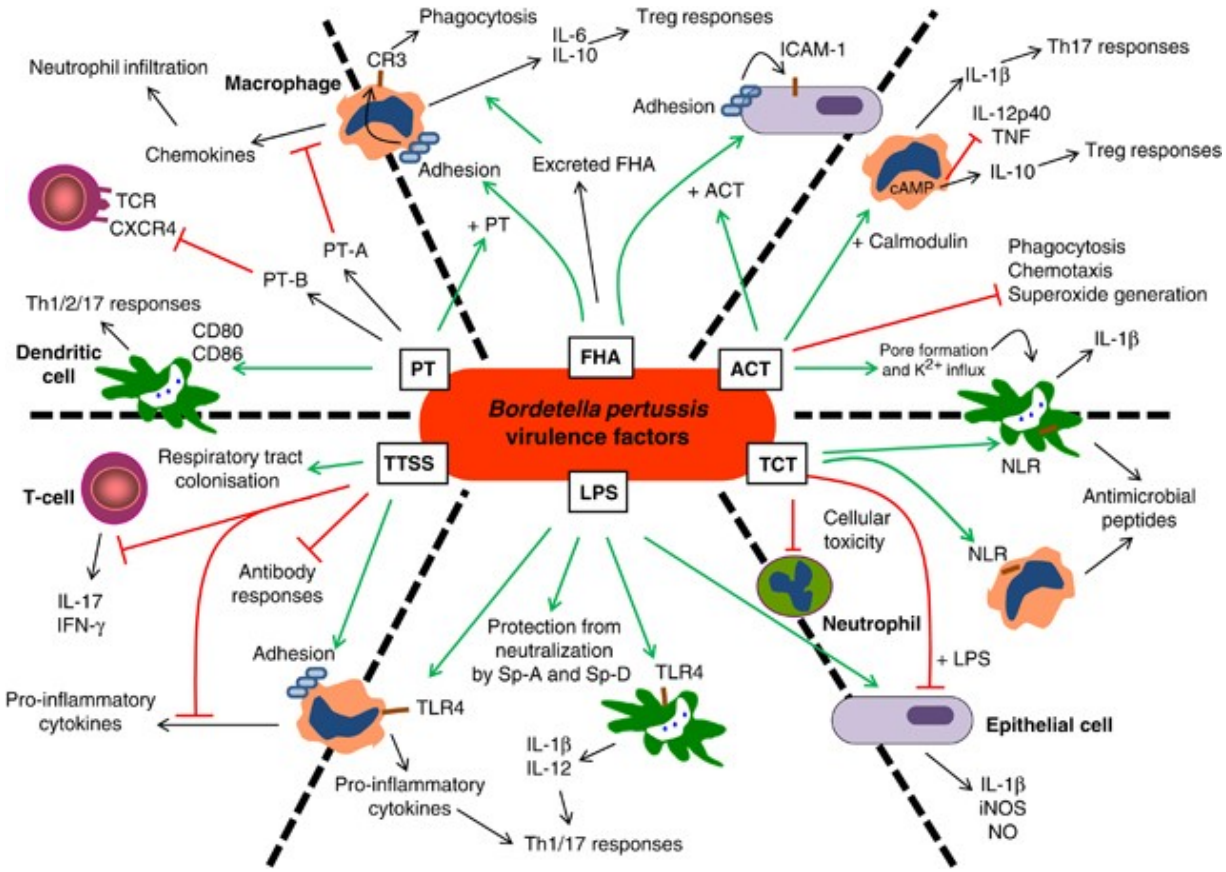

Fig. 10: Immunomodulatory effects of *Bordetella pertussis* virulence factors. The combination of the action of virulence factors like FHA, PT, TCT, ACT, TTSS and LPS enables *B. pertussis* to resist the clearance by the mucociliary escalator and host immune system. Selected effects of individual virulence factors are separated by black dashed lines. Green arrows indicate a positive regulatory effect on the target cell or process. Red bars indicate a negative regulatory effect on the target cell or process. Cells crossing the black dashed lines are targeted by the virulence factor on either side of the line; this layout is for presentation purposes only and does not imply that there is any association between these separate effects. (ICAM-1, intracellular adhesion molecule 1; IL, interleukin; iNOS, inducible nitric oxide synthase; NLR, nucleotide-binding and oligomerization domain (NOD)-like receptor; NO, nitric oxide; SP, surfactant protein; TCR, T-cell receptor; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cells.) Adopted from (Higgs et al., 2012).

Importantly, some of the recent *B. pertussis* isolates from infected individuals do not express all of the virulence factors. With the replacement of the whole cell pertussis vaccine by the acellular pertussis vaccine, containing only few selected antigens, *B. pertussis* adapts to and escapes the vaccine-induced immunity by losing expression of some vaccine antigens/components (Bouchez et al., 2009; Guiso, 2014).

For more details on the effects of virulence factors, pertussis pathogenesis, immunity and vaccination the reader is referred to some of the comprehensive review articles: Locht et al., 2001; Mattoo & Cherry, 2005; Shrivastava & Miller, 2009; Higgs et al., 2012; Fedele et al., 2013; Hewlett et al., 2014; Melvin et al., 2014; Sebo et al., 2014.

4.3.4 Experimental models for pertussis infection

Pertussis pathogenesis has been studied on several animal models such as mice, rabbits, guinea pigs and newborn piglets (Elahi et al., 2007). Even though these models reproduce many aspects of pertussis, none of them fully reflects the pathogenesis of pertussis as it is observed in humans. Obviously, the most important clinical symptom - cough is missing. In addition, typical laboratory animals are not natural *B. pertussis* hosts. However, it was reported previously in some older studies that some of nonhuman primates developed very similar symptoms of pertussis infection as humans (Merkel & Halperin, 2014). Moreover Warfel et al. recently found that a baboon model of pertussis infection seems to reproduce well the typical human course of pertussis disease, including paroxysmal coughing, mucus production and leukocytosis. Moreover, the infection is more severe in young infants than in adult baboons, showing the same age-related correlation as in humans (Warfel & Merkel, 2013). Under certain conditions, which fulfill current ethical and safety standards, Merkel and Halperin also consider using a human challenge model of pertussis (Merkel & Halperin, 2014). Recently, Guevara et al. introduced a good model for *in vitro* studies: primary human airway epithelial cells from human bronchi (Guevara et al., 2016). Hopefully, these new, more relevant models will help to deepen our understanding of pertussis pathogenesis and of the immune response to *B. pertussis* infection, so that a new generation of more efficient pertussis vaccines cab be developed.

4.4 Adenylate cyclase toxin - CyaA

The adenylate cyclase toxin (CyaA, ACT) is a key virulence factor of the whooping cough agent *Bordetella pertussis*. In the murine respiratory model, CyaA was found to be critical for the initiation of lung colonization by the bacteria (Goodwin & Weiss, 1990; Khelef et al., 1992; Harvill et al., 1999). CyaA consists of 1706 amino acids and belongs to the RTX (repeat in toxin) family of bacterial pore-forming toxins (Linhartova et al., 2010). It is a bi-functional toxin possessing two independent activities: an enzymatic cell-invasive adenylate cyclase (AC) domain, consisting of the first 400 residues, whereas a pore-forming RTX 'cytolysin' moiety comprises the remaining 1306 residues.

CyaA primarily targets myeloid phagocytes expressing the $\alpha_M\beta_2$ integrin (CD11b/CD18, CR3 or Mac-1) (Guermonprez et al., 2001) and translocates into their cytosol the adenylate cyclase enzymatic domain. Within the cell, the AC domain binds endogenous calmodulin and catalyses unregulated conversion of cellular ATP to cAMP, a key second messenger signalling molecule (Wolff et al., 1980; Confer & Eaton, 1982; Ladant & Ullmann, 1999). Through elevation of cytosolic cAMP levels the toxin disrupts cellular signaling pathways and significantly modulates host immune responses for the benefit of bacteria (Vojtova et al., 2006; Carbonetti, 2010).

In parallel, the pore-forming conformer of ACT makes cation-selective pores into host cell membrane, promoting efflux of cytosolic potassium ions (Basler et al., 2006; Osickova et al., 2010), which significantly interferes with a host ion homeostasis. Besides, the potassium efflux was shown to contribute to activation of the NALP3 inflammasome complex, which after priming by the LPS signal triggers production of IL-1β by DC (Dunne et al., 2010; Osickova et al., 2010).

CyaA gene and secretion

CyaA toxin expression is controlled by the Bvg two-component system (Scarlato et al., 1991). The *cya* locus consists of five genes, with the *cyaABDE* genes grouped within an operon and the *cyaC* gene being transcribed in an opposite direction. While the *cyaA* gene encodes proCyaA, the other three genes encode the components of a type I secretion system (T1SS) (Glaser et al., 1988; Laoide & Ullman, 1990). The *cyaC* gene then encodes an acyltransferase CyaC, which catalyses the posttranslational palmitoylation of proCyaA (Barry et al., 1991).

CyaA is secreted via the type I secretion system (T1SS). The secretion signal is located at the C-terminal part of the toxin (Sebo & Ladant, 1993). The interaction of the secreted protein with the ABC transporter of T1SS triggers hydrolysis of ATP and provokes power strokes that enable insertion of the secreted protein into the T1SS conduit (Koronakis et al., 2000; Letoffe et al., 1996; Thanabalu et al., 1998). The mechanism of secretion of CyaA (and of other RTX toxins) has just been described in details by Bumba et al. Initially, the calcium-driven assembly of a C terminal capping structure triggers Ca^{2+}-dependent folding of RTX domain. The formation of RTX β-roll structures then ratchets RTX protein translocation out of bacterial cells through the T1SS channel (Bumba et al., 2016).

4.4.1 CyaA structure

The CyaA molecule consists of 1706 residues and consists of two moieties (Fig. 11) (Ladant & Ullmann, 1999). The AC domain (~400 aa) forms the N-terminal part, whereas the pore-forming hemolysin (~1300 aa) forms the C terminal moiety and harbours a hydrophobic pore-forming domain (Basler et al., 2007; Benz et al., 1994; Osickova et al., 1999), an acylation domain (Hackett et al., 1994; Hackett et al., 1995), an RTX domain with characteristic calcium-binding repeats of consensus X-(L/I/F)-X-GG-X-G-(N/D)-D sequence (Rhodes et al., 2001; Rose et al., 1995) and a C-terminal type I secretion signal (Sebo & Ladant, 1993).

In order to become fully functional, the CyaA toxin needs to be post-translationally modified by fatty-acylation. In *Bordetella pertussis* the lysine residues 860 and 983 of CyaA are postranslationally palmitoylated by the toxin acyltransferase CyaC (Hackett et al., 1995, Masin et al., 2005). Further, Ca^{2+} plays an important role in CyaA toxin structure and activity. As it binds to numerous binding sites (~40) within the RTX domain, Ca^{2+} induces a major conformational change of the CyaA molecule that is necessary for folding of the RTX domain and penetration into the cell (Hewlett et al., 1991, Rose et al., 1995, Knapp et al., 2003). Importantly, the crystal structure of the RTX block V (1529-1681) was recently solved (Bumba et al., 2016).

Two functional subdomains (T25 and T18) were identified within the AC domain: the T25 subdomain (1-224 amino acids) represents the adenylate cyclase catalytic site and the T18 subdomain (225-399 amino acids) is responsible for calmodulin binding (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 solved. Four discrete regions of the AC domain were found to bind to the 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 the calmodulin (Guo et al., 2005).

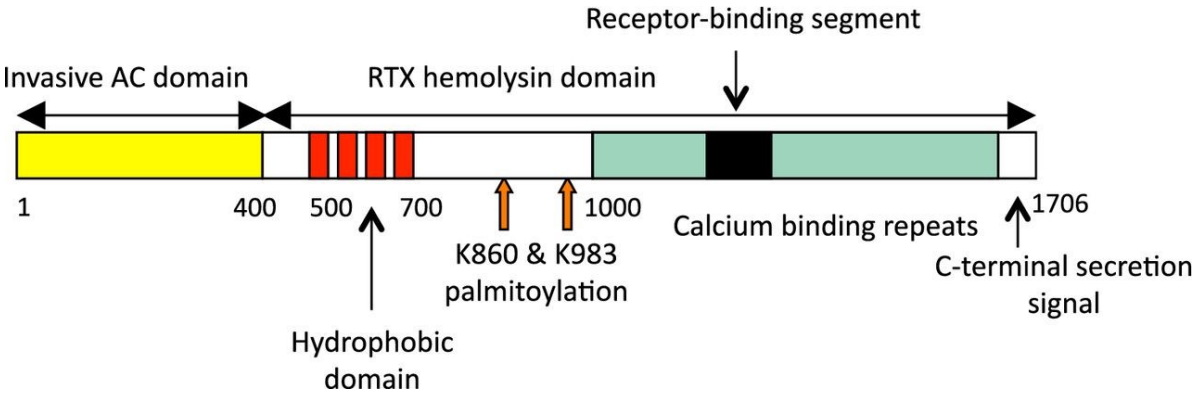

Fig. 11: Structural organization of the CyaA molecule. CyaA (1706 aa) consists of an N-terminal enzymatic adenylate cyclase (AC) domain (~400 residues) and a C-terminal pore-forming RTX hemolysin moiety (~1300 residues). The RTX cytolysin contains several specific sites: a hydrophobic pore-forming domain, sites of post-translational palmitoylation, an integrin binding domain, a calcium binding RTX repeats domain and a C-terminal secretion signal (Masin et al., 2015).

4.4.2 Membrane interaction and penetration of CyaA

The part of the CyaA molecule responsible for binding to its receptor CD11b/CD18 was identified in the RTX domain (residues 1166-1281) (El-Azami-El-Idrissi et al., 2003). It was shown that N-linked glycosylation (Morova et al., 2008; Hasan et al., 2015) and a segment within the CD11b subunit are required for the toxin binding to its integrin receptor (Guermonprez et al., 2001). Recently, Osicka et al. showed that CyaA binds specifically to the segment of CD11b which is located within its residues 614-682 adjacent to its β-propeller domain. This binding site is unique for CD11b and is not present in the two closest relatives of CD11b - the highly homologous CD11a or CD11c subunits of the β2 integrin family (Osicka et al., 2015). CyaA preferentially binds the integrin in a non-activated (bent) conformation (Osicka et al., 2015).

After binding to CD11b/CD18, the toxin either translocates its AC domain across the membrane into cell cytosol, or it forms cation-selective oligomeric pores within cell membrane. The current model of CyaA interaction with cells possessing CD11b/CD18 is schematically shown in Fig. 12. These two activities - the translocation and the pore-forming activity of CyaA would then be to a large extent independent and mutually exclusive. Both precursors would be formed and exist in an equilibrium that can be shifted in either direction by temperature, free calcium concentration, antibody binding, acylation of CyaA, or by specific residue substitutions within the CyaA molecule (Rogel & Hanski, 1992; Betsou et al., 1993; Rose et al., 1995; Gray et al., 1998; Gray et al., 2001; Osickova et al., 1999; Osickova et al., 2010; Rhodes et al., 2001; Basler et al., 2007; Masin et al., 2015).

The process of CyaA translocation across the cytoplasmic membrane of cells appears to be driven by membrane potential (Otero et al.1995; Veneziano et al., 2013). It is independent of membrane permeabillization by CyaA pores (Osickova et al., 2010). The AC domain penetrates the host cell in two sequential steps. First, the insertion of the 'translocation intermediate' permeabilizes the cell membrane for influx of extracellular calcium ions (Fiser et al., 2007), triggering calpain-mediated cleavage of the talin anchor (Bumba et al., 2010). This liberates the integrin-CyaA complex for relocation into lipid rafts, where the lipid environment enables the final step of AC domain translocation (Bumba et al., 2010). Moreover, the calpain then also cleaves the AC domain from the rest of the CyaA molecule (Uribe et al., 2013). Upon translocation, the AC domain binds cytosolic calmodulin (1:1 stoichiometry), resulting in a steep increase of its enzymatic activity and unregulated catalytic conversion of intracellular ATP to cAMP (Ladant & Ullmann, 1999).

In parallel, the CyaA pore precursor oligomerizes and forms small cation selective pores (with a diameter 0.6 to 0.8 nm) that permeabilize host cell membrane (Benz et al., 1994; Vojtova-Vodolanova et al., 2009; Osickova et al., 2010). This leads to potassium efflux and generates a host cell response by activation of several signaling pathways. Recently, Fiser et al. showed that toxin mediated calcium influx and pottasium efflux synergize to cause a significant decrease in endocytic uptake of CyaA molecules from the cytoplasmic membrane. Probably, the Ca^{2+} influx inhibits the uptake of CyaA by the membrane recycling mechanism and thus promotes slower clathrin-dependent endocytic uptake of membrane associated CyaA

molecules (Fiser et al., 2012). This is further slowed down by efflux of potassium ions, which are required for formation of clathrin cups.

CyaA can also penetrate cells lacking the CD11b/CD18 integrin, albeit with a considerably lower efficiency. Nevertheless, CyaA is still able to elevate cAMP levels within these cells (epithelial or other types) to easily detectable levels, due to its very potent enzymatic adenylate cyclase activity (Gordon et al., 1989; Gao et al., 2002).

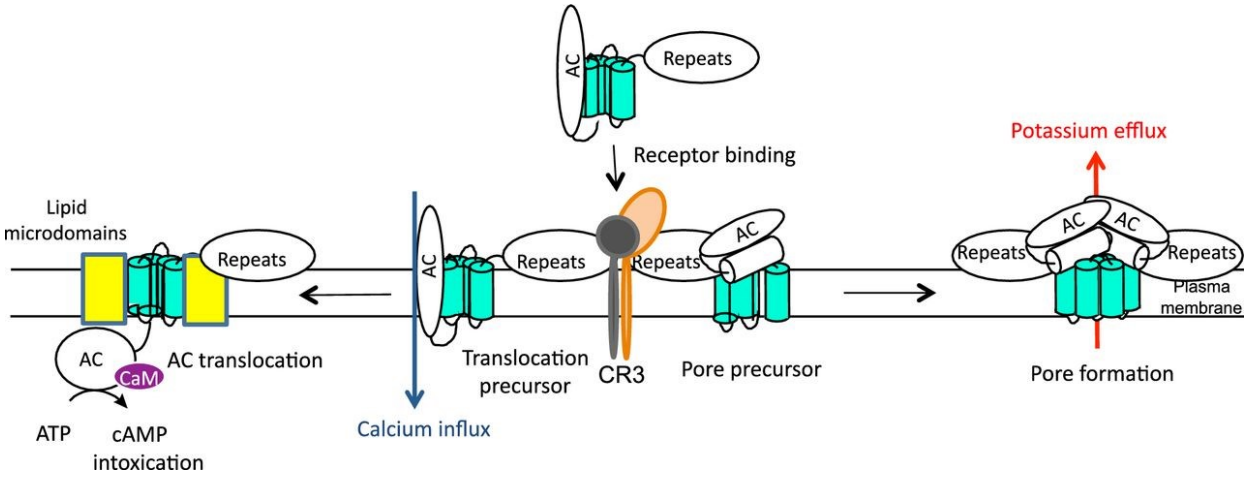

Fig. 12: Model of CyaA action. CyaA targets primarily the host myeloid phagocytes expressing its receptor CD11b/CD18 (CR3, αMβ2 integrin or Mac-1). CyaA appears to have two independent activities: the cell-invasive AC and the pore-forming activity. The current model predicts that two distinct CyaA conformers insert into the host cell membrane. One conformer (pore precursor) oligomerizes to form cation-selective pores that permeabilize the cell membrane for efflux of cytosolic potassium, whereas the other conformer (translocation precursor) causes influx of extracellular calcium ions across cytoplasmic membrane of cells and relocates into lipid rafts, where it translocates its AC domain into cells and thus enables rapid conversion of cytosolic ATP to cAMP (Masin et al., 2015).

4.4.3 Manipulation of host immunity

CyaA is an important virulence factor expressed by all three *Bordetellae* species pathogenic to mammals, i. e. *B. pertussis, B. parapertussis* and *B. bronchiseptica.* As a swift saboteur of host cell functions, CyaA acts on various cell types using several strategies. This leads to manipulation of both innate as well as adaptive immune responses (Vojtova et al., 2006; Sebo et al., 2014).

CyaA plays a prominent role in early recruitment of neutrophils to the site of infection, as observed in the murine respiratory challenge model (Khelef et al., 1994,

Gueirard et al., 1998). On the other hand, CyaA was shown to significantly interfere with innate immunity. In first place, it is a rapid cAMP elevation within phagocytes, which accounts for suppression of their bactericidal functions. It was shown that CyaA through cAMP signaling inhibits reactive oxygen species (ROS) production and impairs opsonophagocytic killing of bacteria in neutrophils and alveolar macrophages (Confer & Eaton, 1982). Moreover, CyaA inhibits both FcR-mediated and complement (CR3)-mediated phagocytosis (Weingart & Weiss, 2000; Mobberley-Schuman et al., 2003; Kamanova et al., 2008). Moreover, also micropinocytosis is impaired due to the CyaA/cAMP signaling and this inhibition is accompanied by massive actin cytoskeleton rearrangements and membrane ruffling due to the inhibition of RhoA (Kamanova et al., 2008). Further, CyaA mediated subversion of chemotaxis and neutrophil extracellular trap formation was also observed (Friedman et al., 1987; Eby et al., 2014). Interestingly, CyaA helps bacteria to survive within the phagosomes (Friedman et al., 1992).

cAMP as a key signaling molecule has profound effects on the downstream signaling pathways triggered by protein kinase A (PKA) and Epac (exchange protein directly activated by cAMP). Recently, Cerny et al. showed that CyaA-mediated PKA activation leads to activation of the tyrosine phosphatase SHP-1 that controls numerous important mechanisms in leukocytes. Interestingly, this inhibits expression of inducible nitric oxide synthase and thus ablates NO production by macrophages, having an important bactericidal function (Cerny et al., 2015). Furthermore, SHP-1 activation results in stabilization of the proapoptotic protein BimEL and Bax activation and thereby induces macrophage apoptosis (Ahmad et al., 2015). This is in agreement with previous reports that CyaA accounts for the induction of apoptosis or necrosis of phagocytes. At higher toxin concentration the toxic effects of cAMP signaling are accompanied by ATP depletion and membrane permeabilization that contribute to cell death (Gueirard et al., 1998; Khelef et al., 1993; Khelef & Guiso, 1995; Bachelet et al., 2002; Basler et al., 2006; Hewlett et al., 2006).

Interestingly, a DNA microarray study showed that CyaA action upregulated transcription of many inflammatory genes in murine macrophages, whereas it downregulated expression of numerous cell-proliferation genes (Cheung et al., 2008).

CyaA interaction with dendritic cells

CyaA is likely to have a good access to airway intraepithelial DC. CyaA was shown to significantly interfere with TLR ligand-induced maturation of dendritic cells, shaping them towards a more tolerogenic phenotype. This manipulation of LPS-stimulated DC is mediated mainly through cAMP signaling of CyaA (Bagley et al., 2002).

CyaA via cAMP mediated signaling interferes with maturation marker expression on the surface of LPS-stimulated DC. CyaA action was shown to enhance levels of the CD80 molecule and decrease levels of CD86, CD40 and CD54 molecules (Skinner et al., 2004; Ross et al., 2004; Boyd et al., 2005). Additionally, CyaA was reported to inhibit production of the proinflammatory cytokines TNF-α and IL-12p70, while enhancing production of the immunosuppressive cytokine IL-10 leading to expansion of Tr1 secreting IL-10. Furthermore, MIP-1α was also shown to be decreased, whereas IL-6 production was potentiated (Njamkepo et al., 2000; Bagley et al., 2002; Ross et al., 2004; Skinner et al., 2004; Boyd et al., 2005; Siciliano et al., 2006; Spensieri et al., 2006). The subversion of the maturation process of DC appears to be caused by CyaA mediated dysregulation of MAPK and IRF signaling (Skinner et al., 2004; Spensieri et al., 2006; Hickey et al., 2008, Fedele et al., 2010).

In parallel, the pore-forming activity of CyaA plays also an important role, as it was recently shown that CyaA-mediated K^+ efflux activates the NALP3 inflammasome and IL-1β production in LPS-primed DC. Subsequently, IL-1β production induced Th17 polarized immune response in mice, which is crucial for the clearance of Bordetella infection from the respiratory tract (Dunne et al., 2010). Similarly, CyaA through its cAMP elevating activity was reported to polarize human dendritic cells towards induction of a Th1/Th17 response, particularly in favour of Th17, through activation of MAPK-pathways (Fedele et al., 2010). Furthermore, it was shown that CyaA accounted for the reduced capacity of human monocytes infected by *B. pertussis* to induce antigen-specific $CD4^+$ T cell proliferation (Boschwitz et al., 1997).

It is plausible to suppose that such CyaA-manipulated DC significantly interfere with the onset of adaptive immune response and thereby delay clearance of bacteria and prolong the persistence of *B. pertussis* infection.

4.4.4 CyaA interaction with the airway epithelial cells

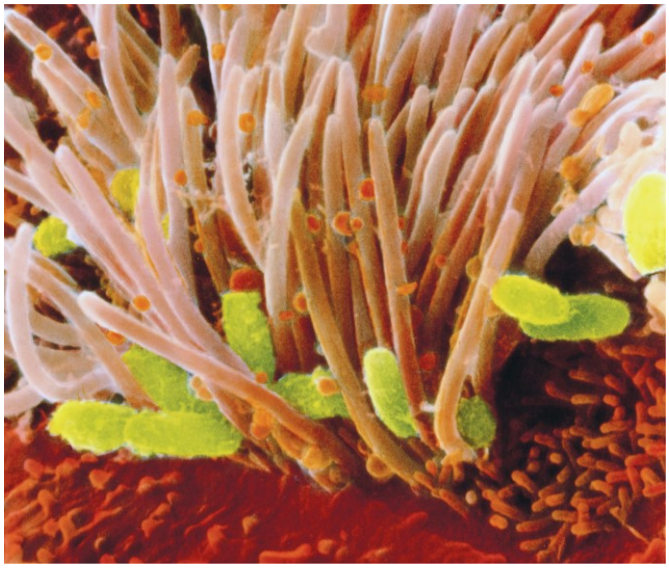

Fig. 13: Bacteria with the airway epithelial cells. *Bordetella pertussis* attaches to cilia in the respiratory tract (Allen, 2013).

An inflammation is often observed in the lungs of patients who died of pertussis. It was shown that the CyaA toxin importantly contributes to numerous pathological effects in the murine model of *Bordetella* infection, such as recruitment of inflammatory leukocytes and an induction of lesions in the lung (Weiss et al., 1984; Khelef et al., 1994; Gueirard et al., 1998). Moreover, CyaA is required for successful colonization by *Bordetella pertussis.* It was shown that bacterial mutants deficient in production of CyaA are not able to colonize tracheal epithelium (Goodwin et al., 1990). Furthermore, the importance of CyaA was recently confirmed by the significant levels of CyaA detected in nasopharyngeal fluids and washes from infected infants and experimentally infected olive baboons (Eby et al., 2013). These authors showed that CyaA levels at host epithelial surface might exceed the concentration of 100 ng/ml (Eby et al., 2013).

Tracheal epithelial cells are one of the first targets of *B. pertussis* during the onset of the infection. Bacteria possess many potent virulence factors which help to breach this first line defence, which creates a mechanical barrier, but also has ability to produce various anti-bacterial agents or different immunomodulators. Interestingly, CyaA was found to inhibit bacterial uptake into human tracheal epithelial cells (Bassinet et al., 2000), while stimulating release of the cytokine IL-6 (Bassinet et al., 2004). CyaA is known to exert various immunomodulatory effects on myeloid cells

possessing the integrin CD11b/CD18 (Vojtova et al., 2006), whereas there is little information on the effects of CyaA on cells lacking this receptor. CyaA was shown to cause cell rounding in several epithelial cell lines due to the adenylate-cyclase activity (Ohnishi et al., 2007). Recently, Eby et al. found that CyaA efficiently translocates across the basolateral membrane of polarized epithelial cells (polarized T84 human colonic adenocarcinoma cell monolayers and human airway epithelial cultures), but surprisingly there was no response to the toxin applied apically (Eby at al., 2010). The basolateral side of T84 epithelial cells seems to be quite sensitive to the CyaA action despite the absence of CD11b/CD18 receptor and the failure to identify another specific membrane receptor of the toxin. The process of intoxication seems to occur independently from post-translational acylation of the toxin, or from host cell membrane potential (Eby et al., 2010), which both were previously reported to be essential prerequisites for toxin entry into cells and action (Basar et al., 2001; El-Azami-El-Idrissi et al., 2003; Otero et al., 1995). CyaA translocation across the basolateral membrane may likely be caused by CyaA interaction with gangliosides located within lipid microdomains of cell membrane (Gordon et al., 1989), followed by internalization through a membrane recycling mechanism. CyaA could also be delivered into polarized epithelial cells via outer membrane vesicles, rather than through direct interaction with the apical cell membrane (Donato et al., 2012). Next, the cAMP signaling could damage the barrier function of airway epithelial layers and provide an opportunity for CyaA to reach epithelial cells from basolateral side, enhancing chloride secretion and mucus production (Eby et al., 2010).

As epithelial cells are capable of producing different cytokines, chemokines and other immunomodulating agents, such as prostaglandins, nitric oxide or antimicrobial peptides (Mayer et al., 2007; Hammad & Lambrecht, 2008; Diamond et al., 2000; Kato & Schleimer, 2007; Schleimer et al., 2007), the action of CyaA and elevation of cAMP levels is likely to contribute to creating a specific environment within the airways, where *B. pertussis* could persist more easily. This seems to be a very interesting and yet poorly understood cross-talk between CyaA manipulated tracheal epithelial cells and the other airway immune cells. Both are quite sensitive to CyaA action, which would lead to a unique regulation of host immune response during *Bordetella pertussis* infection. Nevertheless, how does CyaA manipulate the epithelial cells and what physiological and immunological consequences it has, awaits further elucidation.

4.5 Adenylate cyclase toxoid – CyaA-AC$^-$

CyaA-AC$^-$ is a recombinant toxoid that is devoid of the enzymatic adenylate-cyclase activity of the toxin due to the insertion of a GlySer dipeptide into the ATP binding site of the AC domain (Osicka et al., 2000; Fayolle et al., 2001). The capacity of CyaA to effectively penetrate professional antigen presenting cells (APC), bearing the receptor CD11b/CD18 and to deliver heterologous antigens for both MHC class I and II presentation pathways, allowed to use the CyaA-AC$^-$ toxoid as an antigen delivery tool for induction of specific immune responses against various CD8$^+$ and CD4$^+$ T cells epitopes (Sebo et al., 1995; Osicka et al., 2000; Loucka et al., 2002; Simsova et al., 2004; Adkins et al., 2012). The translocated AC domain, bearing inserted antigens, is chopped to pieces by the proteasome inside the cytosol of antigen-presenting cells. The peptides are then transported by TAP (transporter associated with antigen processing) into the endoplasmic reticulum and are loaded onto MHC I molecules. After transport of these complexes to the cell surface, these can be presented to the CD8$^+$ T cells, thus inducing a specific CD8$^+$ CTL response. In parallel, due to the clathrin-dependent endocytic uptake of the membrane-associated CyaA, antigens carried by CyaA are processed also within the endosomes into peptides and hence reach the MHC II pathway and can activate CD4$^+$ T cells (Loucka et al., 2002; Schlecht et al., 2004), as depicted in detail in Fig 14.

Previously, several permissive sites have been identified in the AC domain into which peptides can be inserted without affecting the cell-invasive activity of the toxoid (Ladant et al., 1992; Osicka et al., 2000). The electrostatic charge of inserted epitopes seems to be critical for the successful AC domain penetration into target cells (Karimova et al., 1998). Moreover, antigens of up to 206 residues in length could be inserted without interference with the ability of the AC domain to be translocated into cell cytosol (Gmira et al., 2001).

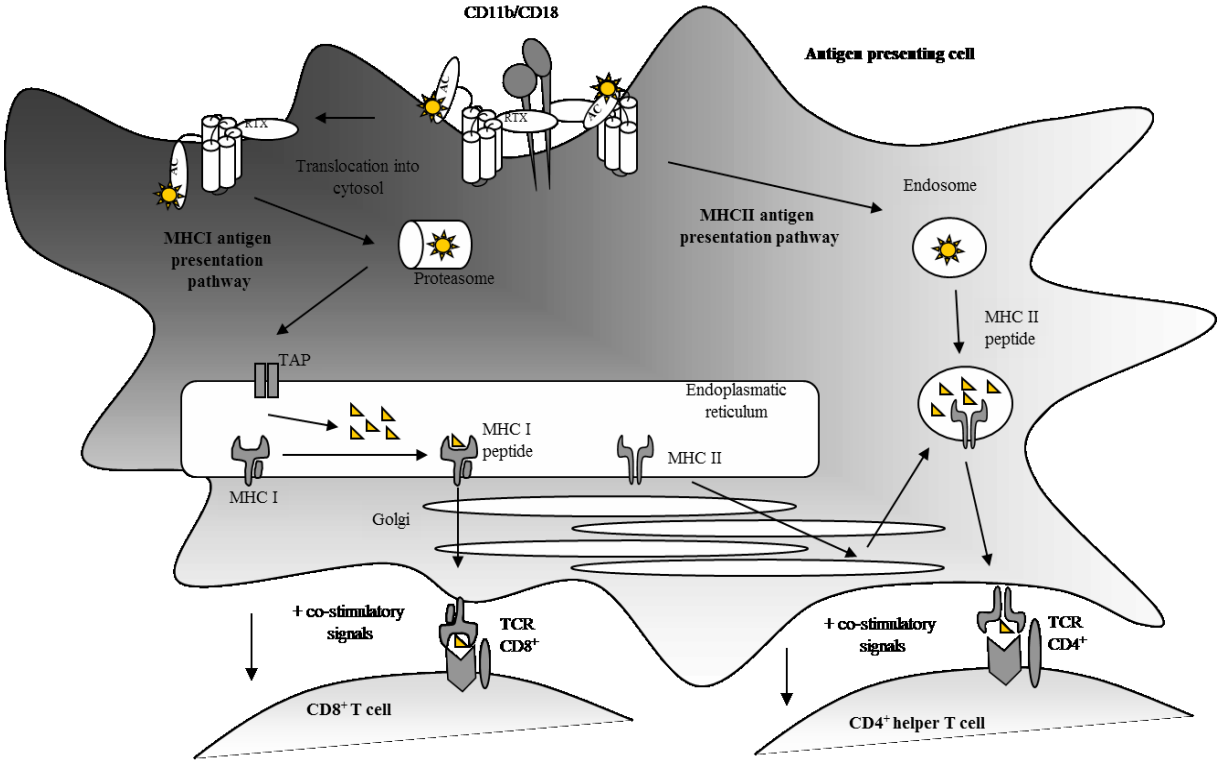

Fig. 14: CyaA as an antigen delivery tool. Numerous heterologous T-cell antigens could be inserted into the enzymatically inactive AC domain of CyaA. The purified CyaA toxoid carrying foreign antigens binds to CD11b/CD18 on the surface of antigen-presenting cells, in particular of dendritic cells. The toxoid penetrates the cellular membrane in two different conformations: the AC domain translocates across the cytoplasmic membrane into cell cytosol, or the molecules form oligomeric membrane channels that permeabilize cells, with the AC domain stuck at the external face of cellular membrane. The translocated AC domain bearing the inserted antigen is processed inside the cytosol by proteasome, the peptide epitopes are then transported by TAP1 into the endoplasmic reticulum and bind to newly synthesized MHC I glycoprotein molecules. The MHC I – peptide complexes are transported to the cell surface and are presented to $CD8^+$ T lymphocytes. In parallel, upon CD11b/CD18 receptor-mediated endocytosis, the membrane-associated molecules with passenger antigens are processed to antigenic peptides in endosomes and bind to MHC II molecules. The MHC II complexes are next exposed on cell surface and presented to $CD4^+$ T cells (Sebo et al., 2014).

4.5.1 CyaA as an antigen delivery tool

CyaA based antigen delivery tools have been extensively used for induction of T cell responses over the past 20 years. Numerous CyaA constructs harbouring

heterologous antigens have been shown to stimulate the immunity against viruses, such as the lymphocytic choriomeningitis virus (LCMV) (Sebo et al., 1995; Fayolle et al., 1996; Saron et al., 1997; Dadaglio et al., 2000; Fayolle et al., 2001), or human immunodeficiency virus (HIV) (Fayolle et al., 1996; Mascarell et al., 2005; Fayolle et al., 2001), the cytomegalovirus (CMV) (Jelinek et al., 2011) and influenza virus A (Stanekova et al., 2013). The first evidence that CyaA has the capacity to deliver exogenous antigens and stimulate strong CTL responses *in vivo* was shown in mice that were immunized with recombinant CyaA toxins carrying $CD8^+$ T cell epitopes from LCMV and HIV (Fayolle et al., 1996; Saron et al., 1997). Moreover, mice immunized by the CyaA construct with the LCMV epitope were protected against lethal challenge with LCMV (Saron et al., 1997; Fayolle et al., 2001). Heterologous prime/boost immunization with CyaA toxoid carrying an epitope from the parasite *Plasmodium berghei* elicited protection in mice (Tartz et al., 2006; Tartz et al., 2008). CyaA could be also used as a useful diagnostic tool for the detection of latent tuberculosis (Vordermeier et al., 2004; Wilkinson et al., 2005; Anderson et al., 2006). CyaA toxoids delivering antigens for Mycobacterium tuberculosis failed, however, to induce protective immunity against tuberculosis in mice (Majlessi et al., 2006; Hervas-Stubbs et al., 2006). Immunization of mice with CyaA toxoids bearing CTLs epitope provided efficient protective and therapeutic antitumor immunity (Fayolle et al., 1999). Most importantly, CyaA-AC^- was demonstrated to elicit both protective and therapeutic immune responses against HPV-16(18)-induced tumors (Preville et al., 2005; Mackova et al., 2006) and melanoma in mice (Dadaglio et al., 2003). The phase I clinical trial testing CyaA for the delivery of the E7 oncoantigen from HPV 16 and 18 for immunotherapy of cervical cancer proved safety, immunogenicity and clinical efficacy (Van Damme et al., 2016). However, phase II clinical trial failed to prove clinical efficacy of the vaccine (www.genticel.com). This will need to be repeated with an improved vaccine design. Besides, there is another Phase I/II clinical trial underway in which CyaA is delivering a tyrosinase epitope into dendritic cells of patients with advanced metastatic melanoma (www.centerwach.com).

The summary of studies which exploited CyaA for antigen delivery purposes are presented in Table 2. This issue has been reviewed in more details elsewhere (Simsova et al., 2004; Adkins et al., 2012).

Table 2: Adenylate cyclase toxin (CyaA) of *B. pertussis* as a vector for an antigen delivery (adopted from Adkins et al., 2012, see references in the review)

Antigen	Origin	Carrier	Immune response (Model; CMR)[a]	Protection in animal model[b]
$NP_{118-132}$	Nucleoprotein of lymphocytic choriomeningitis virus (LCMV)	CyaA wt[c]	*in vitro* CTL-mediated target cell lysis	ND
		CyaA wt CyaA-AC$^-$	BALB/c; CTL	ND
		CyaA-wt CyaA-AC$^-$	BALB/c; CTL	+
		CyaA-AC$^-$	BALB/c; CTL; $CD4^+$ T cells (Th1)	ND
		CyaA-AC$^-_{cys}$	BMDC, splenocytes, C57BL/6, BALB/c; CTL	ND
		CyaA-AC$^-$	BALB/c; CTL multiepitope containing also $OVA_{257-264}$ and $V3_{316-327}$	+ (LCMV)
$OVA_{257-264}$	Chicken egg ovalbumin	CyaA-AC$^-$	C57BL/6; CTL	+
		CyaA-AC$^-$	$CD8^+$ T cell hybridoma (B3Z)	ND
		CyaA-AC$^-_{cys}$	BMDC, splenocytes, C57BL/6, BALB/c; CTL	ND
$MalE_{126-140}$	Maltose-binding protein of *E. coli*	CyaA-AC$^-$	C57BL/6, BALB/c; $CD4^+$ T cell hybridomas (CRMC3 and FBCD1)	ND
$V3_{316-327}$	HIV	CyaA wt CyaA-AC$^-$	BALB/c; CTL	ND
Tat		CyaA-AC$^-$	BALB/c, CTL, $CD4^+$ T cells (Th1); neutralizing Ab	ND
$Tyr_{369-377}$ GnT-V	Melanoma epitopes: Tyrosinase (Tyr) N-acetylglucosaminyl-transferase V (GnT-V)	CyaA-AC$^-$	HHD mice expressing HLA*0201; human DC; CTL	ND
E7, $E7_{49-57}$ $E7_{\Delta 30-42}$	Human papillomavirus 16 (HPV16)	CyaA-AC$^-$	C57BL/6; CTL, $CD4^+$ T cells (Th1)	+
		CyaA-AC$^-$	BMDC, C57BL/6	+
ESAT-6 CFP10	*M. tuberculosis*	CyaA-AC$^-$	IFN-γ production by bovine PBMC; CFP10-specific $CD4^+$T cell line	ND
		CyaA-AC$^-$	IFN-γ production by human PBMC; $CD8^+$ and $CD4^+$ T cells	ND
Ag85A		CyaA-AC$^-$	BMDC, BALB/c , C57BL/6; $CD4^+$ T cells (Th1)	-
TB10.4 $TB10.4_{74-88}$ $TB10.4.4_{20-28}$ $TB10.4.4_{15-33}$		CyaA-AC$^-$	BMDC, BALB/c; CTL, $CD4^+$ T cells	-
$CSP_{245-253}$	*P. berghei*	CyaA-AC$^-$	BALB/c; CTL	+/-
		CyaA-AC$^-$	BALB/c; CTL	+

[a] Abbreviations: CMR, cell-mediated response; CTL, cytotoxic T lymphocytes; Ab, antibody response; Cys, cysteine; BMDC, bone marrow-derived dendritic cells; PBMC, peripheral blood mononuclear cells

[b] +, 100 % protection; -, no protection; +/-, partial protection; ND, not determined

[c] CyaA wt, wild type enzymatically active CyaA toxin; CyaA-AC$^-$, CyaA toxoid with ablated adenylate cyclase activity

4.5.2 Adjuvant activity of CyaA-AC$^-$

CyaA is not only a virulence-associated factor, but also a potent immunoprotective antigen. The immunogenic properties of CyaA were first detected as the presence of high-titre anti-CyaA antibodies in either pertussis patients or in vaccinated infants and adults (Farfel et al., 1990; Arciniega et al., 1991). Vaccination with CyaA purified from *Bordetella pertussis* was able to protect infant mice against the lethal challenge at 14 days after vaccination and it also shortened the period of bacterial colonization (Guiso et al., 1989; Guiso et al., 1991). Native CyaA prepared from *Bordetella pertussis,* as well as recombinant CyaA prepared from *Escherichia coli,* exhibited adjuvant and protective properties when used as vaccine. The protective antigen capacity of CyaA correlated with its post-translational activation by acylation (Betsou et al., 1993; Hormozi et al., 1999). The protective anti-CyaA antibodies were directed to the last 800 residues of the protein, probably recognizing conformational epitopes formed in the C-terminal RTX domain (Betsou et al., 1995; Wang et al., 2015). Interestingly, the reported adjuvant ability of CyaA was observed after co-administration of ovalbumin, which resulted in the presence of high amount of anti-ovalbumin IgG antibodies accompanied also by anti-CyaA antibodies (Hormozi et al., 1999).

Besides the ability to deliver antigens into professional antigen presenting cells (APC), the potency of CyaA-AC$^-$ or previously used CyaA in inducing antigen-specific T cell responses appears to depend on the adjuvant capacity of the toxin/toxoid. Interestingly, the detoxified form of CyaA toxin, CyaA-AC$^-$, exhibited even better adjuvant properties than the wild type toxin. Intraperitoneal co-administration of the CyaA-AC$^-$ toxoid enhanced serum IgG antibody response to the components of the acellular pertussis vaccine (PT/dPT, FHA and PRN) and increased the nitric oxide and IFN-γ production from peritoneal macrophages and spleen cells of vaccinated animals collected two weeks post-immunisation. Nevertheless, this potent adjuvant effect of CyaA-AC$^-$ was not accompanied by an increased protection of mice against the aerosol challenge with *B. pertussis*, though there was at least an improved protection of mice after the intranasal challenge with *B. parapertussis* (MacDonald-Fyall et al., 2004).

Both CyaA and CyaA-AC$^-$ could stimulate mucosal as well as systemic immune response when co-administered with ovalbumine by the intranasal route in

mice, with the second one reported to be more effective (Orr et al., 2007). Enhanced anti-ovalbumine IgG and IgA antibody response in the serum and anti-ovalbumine IgA responses in the lung and nasal secretions was observed after intranasal co-administration of CyaA/CyaA-AC$^-$ with ovalbumin (OVA) compared to immunization with OVA alone. In addition, enhanced priming of OVA-specific T cells was also detected. Similarly, higher antibody secretion against co-administered CyaA/CyaA-AC- with pertactin was determined, leading to significantly increased protection of mice against *B. pertussis* challenge compared to protection obtained upon vaccination with pertactin alone (Orr et al., 2007).

Furthermore, Cheung et al. documented that only the CyaA toxoid and not the toxin significantly enhanced the potency of a commercial aP vaccine to confer protection against the intranasal challenge by *B. pertussis* in a murine model. This was facilitated probably by an increased Th1 and Th2 immune response to *B. pertussis* antigens included in the aP vaccine. Moreover, an increased level of IgG2a antibody to pertactin and enhanced IL-5, IL-6, IFN-γ and GM-CSF secretion by splenocytes and increased NO production by restimulated macrophages was observed, in comparison to the immunization with the CyaA toxoid or aP alone (Cheung et al., 2006).

The CyaA-AC$^-$ toxoid lacks the adenylate cyclase activity, but it is still able to act as an adjuvant and is even more potent than the enzymatically active toxin (MacDonald-Fyall et al., 2004; Orr et al., 2007; Cheung et al., 2006). Moreover, Cya-AC$^-$ was shown to induce rather Th1-polarized immune responses (Dadaglio et al., 2003; Ross et al., 2004; Mascarell et al., 2005), which is more characteristic for previously used wP vaccines as well as for the pertussis infection, by difference to mixed Th1 and Th2 response being obtained after vaccination of children with aP vaccine (Ausiello et al., 1997; Ryan et al., 1998). Therefore, Cya-AC$^-$ is a promising antigen candidate for inclusion into the next generation of aP vaccines.

5. AIMS

Despite many studies on the CyaA toxin and toxoid, the mechanisms of their action are still poorly understood. Therefore we wanted to address several issues, particularly concerning the adjuvant effect of the toxoid and its capacity to deliver antigens and induce specific immune responses against selected inserted epitopes. Besides, we wanted to examine the role of CyaA toxin (and its adenylate cyclase and pore-forming activity) under infectious conditions.

Hence, our experimental work had the following aims:

- Identify the mechanism of action of the adenylate cyclase toxoid which is responsible for its adjuvanticity. Characterize the CyaA-AC$^-$-induced maturation, migratory and T cell stimulatory capacity of DC.
- Characterize the antigen delivery capacity of mutants of adenylate cyclase toxin with shortened AC domain, in order to determine which parts of the AC domain are necessary for its translocation across the cell plasma membrane.
- Prepare and purify the recombinant adenylate cyclase toxoids bearing the CD8$^+$ and CD4$^+$ T cells epitopes of the HA2 subunit of hemagglutinin of influenza A viruses. These toxoids will further be tested in mouse model for the ability to induce effective protection against different variants of influenza A viruses.
- Determine how active adenylate cyclase toxin modulates the functions of LPS-stimulated dendritic cells.
- Test if the pore-forming activity of adenylate cyclase toxin contributes to virulence of *Bordetella pertussis* during infection (lethality and pathogenesis)

6. RESULTS

6.1 Publications

Svedova, M., Masin, J., Fiser, R., Cerny, O., Tomala, J., Freudenberg, M., Tuckova, L., Kovar, M., Dadaglio, G., Adkins, I., & Sebo, P. (2016). Pore-formation by adenylate cyclase toxoid activates dendritic cells to prime CD8+ and CD4+ T cells. *Immunol Cell Biol, 94*(4), 322-333. doi:10.1038/icb.2015.87

Adkins, I., Kamanova, J., Kocourkova, A., **Svedova, M.**, Tomala, J., Janova, H., Masin, J., Chladkova, B., Bumba, L., Kovar, M., Ross, P. J., Tuckova, L., Spisek, R., Mills, K. H., & Sebo, P. (2014). Bordetella adenylate cyclase toxin differentially modulates toll-like receptor-stimulated activation, migration and T cell stimulatory capacity of dendritic cells. *PLoS One, 9*(8), e104064. doi:10.1371/journal.pone.0104064

Stanekova, Z., Adkins, I., **Kosova, M.**, Janulikova, J., Sebo, P., & Vareckova, E. (2013). Heterosubtypic protection against influenza A induced by adenylate cyclase toxoids delivering conserved HA2 subunit of hemagglutinin. *Antiviral Res, 97*(1), 24-35. doi:10.1016/j.antiviral.2012.09.008

Holubova, J., Kamanova, J., Jelinek, J., Tomala, J., Masin, J., **Kosova, M.**, Stanek, O., Bumba, L., Michalek, J., Kovar, M., & Sebo, P. (2012). Delivery of large heterologous polypeptides across the cytoplasmic membrane of antigen-presenting cells by the Bordetella RTX hemolysin moiety lacking the adenylyl cyclase domain. *Infect Immun, 80*(3), 1181-1192. doi:10.1128/IAI.05711-11

Skopova K., Tomalova B., Kanchev I., Rossmann P., **Svedova M.**, Adkins I., Bibova I., Tomala J., Masin J., Guiso N., Osicka R., Sedlacek R., Kovar M., Sebo P. (2017) Hemolytic activity of adenylate cyclase toxin is not required for lung colonization and subversion of dendritic cell function but contributes to virulence of *Bordetella pertussis*. *Infect Immun,* in press

Adkins, I., Holubova, J., **Kosova, M.,** & Sadilkova, L. (2012). Bacteria and their toxins tamed for immunotherapy. *Curr Pharm Biotechnol, 13*(8), 1446-1473.

6.2 PUBLICATION 1

Svedova, M., Masin, J., Fiser, R., Cerny, O., Tomala, J., Freudenberg, M., Tuckova, L., Kovar, M., Dadaglio, G., Adkins, I., & Sebo, P. (2016). Pore-formation by adenylate cyclase toxoid activates dendritic cells to prime CD8+ and CD4+ T cells. *Immunol Cell Biol, 94*(4), 322-333. doi:10.1038/icb.2015.87

Bordetella adenylate cyclase toxin (CyaA) is a bi-functional toxin with an enzymatic AC domain and a hemolytic (pore-forming) RTX domain. It targets primarily phagocytic cells expressing CD11b/CD18 receptor (CR3, $\alpha_M\beta_2$ integrin) and has the ability to penetrate into cell membrane and deliver foreign antigens inserted within its AC domain into cell cytosol. In parallel, CyaA can form cation-selective pores within the cell membrane and hence cause potassium leakage from cell cytosol. Enzymatically inactivated CyaA-AC$^-$ toxoid has been used as an antigen delivery tool over the past 20 years. Currently, CyaA-derived toxoids are under evaluation in clinical trials as immunotherapeutic vaccines for treatment of metastatic melanoma and of the papilloma virus-induced cervical cancer. One of the most intriguing ability of CyaA toxoid is the capacity to act as adjuvant. However, this capacity remained poorly understood. We supposed that CyaA toxoid might exert its adjuvant activity through targeting and activation of DC. Therefore, we characterized here the CyaA toxoid-induced DC maturation profile together with the migratory and T cell stimulatory capacity of toxoid-exposed DC. Further, we focused on deciphering the possible mechanism of toxoid adjuvanticity by using a set of CyaA mutants with reduced or enhanced pore-forming activity.

We show that it is the pore-forming activity of the toxoid which is crucial for its adjuvant effect, causing activation of mitogen-activated protein kinases JNK and p38 and leading to the maturation of CyaA-AC$^-$-treated dendritic cells. We further showed that such maturation is independent of Toll-like receptors and inflammasome signaling, while it is accompanied by up-regulated expression of maturation markers, increased production of interleukin 6, of the chemokines KC and LIX, of granulocyte colony stimulating factor and prostaglandin E and stimulation of chemotactic migration of DC. This was confirmed also *in vivo*, as CyaA-AC$^-$ injected intravenously into mice induced maturation of splenic DC. Moreover, such stimulated DC exhibited capacity to induce CD4$^+$ and CD8$^+$ T cell responses *in vitro* and *in vivo*. This reveals

a novel self-adjuvanting capacity of the CyaA-AC^{-} toxoid that is currently under clinical evaluation as a tool for the delivery of immunotherapeutic anti-cancer CD8^{+} T cell vaccines into DC.

My contribution: Preparation of recombinant CyaA toxoids, generation of BMDC, I conducted experiments characterizing maturation state of DC - expression of maturation markers by flow cytometry and production of inflammatory molecules; maturation experiments with BMDC from knock-out mice, migration experiments, PGE2 production by ELISA, activation of MAPK by western blot, experiments with MAPK inhibitors, *in vivo* maturation of splenic DC, helped in experiments characterizing ability of DC to stimulate T cells - T cell expansion and T cell response experiments, helped to write draft of manuscript.

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www.nature.com/icb

ORIGINAL ARTICLE

Pore-formation by adenylate cyclase toxoid activates dendritic cells to prime CD8$^+$ and CD4$^+$ T cells

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The adenylate cyclase toxin-hemolysin (CyaA) of *Bordetella pertussis* is a bi-functional leukotoxin. It penetrates myeloid phagocytes expressing the complement receptor 3 and delivers into their cytosol its N-terminal adenylate cyclase enzyme domain (~400 residues). In parallel, ~1300 residue-long RTX hemolysin moiety of CyaA forms cation-selective pores and permeabilizes target cell membrane for efflux of cytosolic potassium ions. The non-enzymatic CyaA-AC$^-$ toxoid, has repeatedly been successfully exploited as an antigen delivery tool for stimulation of adaptive T-cell immune responses. We show that the pore-forming activity confers on the CyaA-AC$^-$ toxoid a capacity to trigger Toll-like receptor and inflammasome signaling-independent maturation of CD11b-expressing dendritic cells (DC). The DC maturation-inducing potency of mutant toxoid variants *in vitro* reflected their specifically enhanced or reduced pore-forming activity and K$^+$ efflux. The toxoid-induced *in vitro* phenotypic maturation of DC involved the activity of mitogen activated protein kinases p38 and JNK and comprised increased expression of maturation markers, interleukin 6, chemokines KC and LIX and granulocyte-colony-stimulating factor secretion, prostaglandin E2 production and enhancement of chemotactic migration of DC. Moreover, i.v. injected toxoids induced maturation of splenic DC in function of their cell-permeabilizing capacity. Similarly, the capacity of DC to stimulate CD8$^+$ and CD4$^+$ T-cell responses *in vitro* and *in vivo* was dependent on the pore-forming activity of CyaA-AC$^-$. This reveals a novel self-adjuvanting capacity of the CyaA-AC$^-$ toxoid that is currently under clinical evaluation as a tool for delivery of immunotherapeutic anti-cancer CD8$^+$ T-cell vaccines into DC.

Immunology and Cell Biology (2016) **94,** 322–333; doi:10.1038/icb.2015.87

The adenylate cyclase toxin-hemolysin (CyaA) is a 1706 residue long bi-functional leukotoxin that has a central role in *Bordetella pertussis* virulence, particularly in the early phases of airway colonization.[1,2] CyaA is endowed with a cell-invasive N-terminal adenylate cyclase (AC) enzyme domain (~400 residues) that is fused to a pore-forming RTX (repeat in toxin) 'cytolysin' (Hly) moiety of ~1300 residues. The Hly portion binds the complement receptor 3 (CR3, known as the $\alpha_M\beta_2$ integrin CD11b/CD18 or Mac-1) and penetrates the membrane of myeloid phagocytes.[3,4] CyaA insertion into the cytoplasmic membrane of phagocytes then triggers influx of calcium ions[5] that activates calpain-mediated cleavage of talin.[6] This mobilizes the CyaA–CR3 complex for relocation into membrane microdomains (lipid rafts), from where the translocation of the AC domain across cellular membrane is completed.[6,7] Inside cells the AC enzyme binds calmodulin and catalyzes unregulated conversion of ATP into the key signaling molecule cAMP, which ablates bactericidal activities of phagocytes.[4] In parallel, the Hly moiety of CyaA, which is functionally independent of the invasive AC domain, forms cation-selective pores and permeabilizes cellular membrane for efflux of cytosolic potassium ions from cells.[7,8] The pore-forming activity of CyaA-AC$^-$ was then shown to synergize with Toll-like receptor (TLR) signaling in promoting NALP3 inflammasome complex assembly and IL-1β secretion in dendritic cells (DC).[9]

The cell-invasive AC domain of CyaA was shown to accommodate large polypeptide inserts without losing its capacity to penetrate cells.[10,11] Therefore, non-enzymatic CyaA-AC$^-$ toxoids, having a variety of heterologous antigens inserted into, or in place, of the AC domain of CyaA, have been extensively exploited over the past two decades for delivery of antigens into CD11b$^+$ DC for processing and antigenic presentation to T cells. These recombinant CyaA-AC$^-$ constructs were, indeed, shown to induce potent antigen-specific cytotoxic CD8$^+$ T-lymphocyte responses against various viruses (for example, HIV, CMV, LCMV, HPV or influenza), bacteria (*Myobacterium tuberculosis*), parasites (*Plasmodium berghei*), or tumors (melanoma or HPV-induced).[12,13] Building on these studies, three CyaA-derived toxoids currently reached the stage of phase I and II

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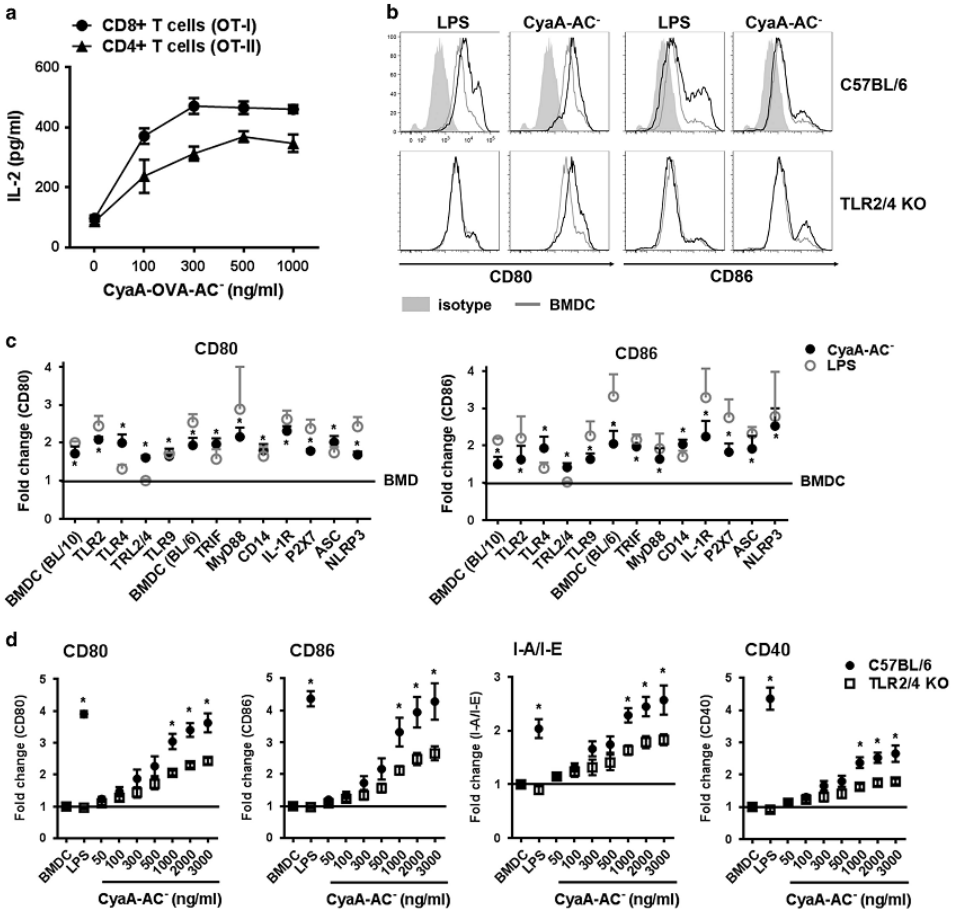

Figure 1 Low concentration of CyaA-AC$^-$ toxoid triggers CD80 and CD86 expression on BMDC *in vitro* by a mechanism that does not involve TLR$^-$ or inflammasome-mediated signaling. (**a**) BMDC (1×10^6 ml^{-1}) were treated with indicated concentrations of CyaA-OVA-AC$^-$ toxoid carrying ovalbumin epitopes for MHC I and MHC II for 4 h, washed and subsequently added to OVA-specific CD8$^+$ T cells (OT-I) or CD4$^+$ T cells (OT-II). IL-2 was detected by enzyme-linked immunosorbent assay in cell culture supernatant after 3 days. Values are representative of three independent experiments. (**b** and **c**) BMDC (1×10^6 ml^{-1}) prepared from indicated knockout mice were incubated with LPS (100 ng ml^{-1}) or CyaA-AC$^-$ toxoid (300 ng ml^{-1}) for 24 h. The expression of CD80 and CD86 molecules on CD11c$^+$Hoechst negative cells was determined by flow cytometry. (**b**) Histograms are representative of $n=3$. Gray histogram represents isotype control, gray line represents non-treated BMDC, black line represents LPS or CyaA-AC$^-$ treatment. (**c**) Values represent the means ± s.e. from at least three independent experiments expressed as fold change of marker expression on mock-treated BMDC according to an appropriate genetic background from C57BL/6 or C57BL/10 mice (*$P<0.05$). (**d**) CyaA-AC$^-$ concentration-dependent induction of maturation of DC generated from wt C57BL/6 and TLR2/4 KO mice. Values represent the means ± s.e. expressed as fold change of marker expression on mock-treated BMDC for each mouse strain (*$P<0.05$).

clinical trials in humans and are being evaluated as immunotherapeutic cytotoxic CD8$^+$ T-lymphocyte vaccines for treatment of metastatic melanoma and papilloma virus-induced cervical cancer, respectively.

Besides inducing T-cell immune responses through delivery of antigens for presentation into DC, the CyaA-AC$^-$ toxoid was also shown to possess an inherent adjuvant capacity. CyaA-AC$^-$ was shown to enhance antibody and T-cell responses to co-administered *B. pertussis* antigens.[14,15] Moreover, co-administration of lipopolysaccharides (LPS)-free CyaA-AC$^-$ with a diluted commercial pertussis vaccine was found to shift the polarization of the resulting immune response from a typical Th2 type to a mixed Th1/Th2 type of response.[16] This importantly improved the resulting protection of mice against intranasal challenge by *B. pertussis*, well beyond the contribution of anti-CyaA antibodies.[16] These observations indicated that the CyaA-AC$^-$ toxoid might exert its adjuvant activity through targeting and activation of DC. Recently Dadaglio *et al.* showed that adjuvant activity of high doses of LPS-free CyaA-AC$^-$ toxoid on DC

depends on TLR4/TRIF signaling. The $CD8^+$ T-lymphocyte response was, however, not completely abolished in $TLR4^{-/-}$ and $TRIF^{-/-}$ mice *in vivo*,[17] suggesting that CyaA-AC^- may employ an additional adjuvant mechanism on DC to potentiate their capacity to induce T-cell responses. Here we show that specifically at low toxoid concentrations, the $CyaA^-$ exerts a novel intrinsic adjuvant activity on DC *in vitro* and *in vivo*. This is independent of TLR- and inflammasome-mediated signaling and depends on the pore-forming activity of the toxoid that causes K^+ efflux-mediated activation of p38 and JNK mitogen activated protein kinases (MAPKs).

RESULTS

At low concentration the CyaA-AC^- toxoid triggers CD80 and CD86 expression on BMDC *in vitro* by a mechanism that does not involve TLR or inflammasome-mediated signaling

We first tested a minimal toxoid concentration required for efficient antigen delivery into DC for T-cell stimulation *in vitro*, using toxoids that contained <0.12 EU of LPS per 1 μg of toxoid (<12 pg LPS/ 1 μg of CyaA-AC^-). Murine bone marrow-derived DC (BMDC) were incubated for 4 h with various concentrations (100–1000 ng ml^{-1}) of the CyaA-OVA-AC^- toxoid carrying ovalbumin epitopes presented on MHC class I and II molecules. Cells were then washed, incubated with OVA-specific naïve $CD8^+$ (OT-I) or $CD4^+$ (OT-II) T cells and IL-2 production in supernatants of DC and T-cell co-cultures was measured after 3 days. As shown in Figure 1a, BMDC incubated with as low concentration of CyaA-OVA-AC^- as 300 ng ml^{-1} displayed similar $CD8^+$ and $CD4^+$ T-cell stimulatory capacity as BMDC incubated with the higher toxoid concentration of 500 or 1000 ng ml^{-1}. Given the very low endotoxin content of the toxoid preparations, this indicated that it was the activity of the toxoid itself that conferred a DC maturation-dependent T-cell stimulatory capacity on the BMDC.

As CyaA-AC^- at the highest concentration of 1000 ng ml^{-1} induced DC maturation (data not shown), we examined in detail whether as little as 300 ng ml^{-1} of CyaA-AC^- would induce DC maturation and what signaling pathway(s) might be triggered by the toxoid. Towards this aim we used a set of BMDC derived from mice deficient in CD14, TLR 2, 4, 2/4 and 9, TLR adapters TRIF (TIR-domain-containing adapter-inducing interferon-β) and MyD88 (myeloid differentiation primary response gene 88), or deficient in molecules participating in inflammasome and IL-1 signaling such as IL-1 R, P2X7, ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (NOD-like receptor family, pyrin domain containing 3, respectively. The BMDC were treated with 300 ng ml^{-1} of CyaA-AC^- for 24 h and analyzed by flow cytometry, using CD80 and CD86 expression as markers of maturation. When added into BMDC suspensions this CyaA-AC^- dose (300 ng ml^{-1}) yielded a final endotoxin concentration of <0.036 EU ml^{-1} (for example, <3.6 pg ml^{-1} of LPS). As a positive control DC maturation stimulus, a dose of 100 ng ml^{-1} of *Escherichia coli* LPS was used (Figure 1b). As however shown in Figure 1c, the used LPS contained also traces of TLR2 ligands, inducing to some extent also maturation of TLR4-deficient BMDC. It, however, did not trigger maturation of BMDC deficient for both the TLR2 and TLR4 (TLR2/4) receptors (Figures 1b and c). In contrast, the highly purified CyaA-AC^- toxoid (300 ng ml^{-1}) triggered induction of CD80 and CD86 marker expression not only in the TLR2/4 knockout BMDC, that were insensitive to LPS (Figures 1b and c), but promoted also maturation of all other used knockout BMDC that were deficient in the other TLR or inflammasome signaling components (Figure 1c). This indicated that the CyaA-AC^- toxoid used at 300 ng ml^{-1} possessed an intrinsic DC-activating capacity that did not depend on TLR- or inflammasome-mediated signaling pathways.

As, however, shown in Figure 1d, at higher toxoid concentrations ranging from 1 to 3 μg ml^{-1}, a significantly higher expression of CD80, CD86, I-A/I-E and CD40 maturation markers was observed on DC generated from wild type mice (C57BL/6) than on DC from TLR2/4 KO mice. These results, hence, show that CyaA-AC^- induces both TLR-dependent and TLR-independent DC maturation. The TLR-dependent maturation is then particularly triggered at high toxoid concentrations of $\geqslant 1$ μg ml^{-1}, presumably via the TLR4/TRIF signaling mechanism reported previously.[17]

Pore-forming activity-dependent K^+ efflux accounts for phenotypic maturation of BMDC exposed to low concentrations of the CyaA-AC^- toxoid

The enzymatically inactive CyaA-AC^- toxoids still possess the capacity to form pores in membranes of CD11b-expressing cells and permeabilizes cells for efflux of cytosolic potassium ions.[9,18,19] As K^+ efflux was shown to induce DC maturation,[20] we examined whether it was the cell-permeabilizing activity of the CyaA-AC^- toxoid at 300 ng ml^{-1} that induced maturation of BMDC. Towards this aim, advantage was taken of a previously characterized set of CyaA-AC^- toxoids that have the specific pore-forming activity selectively enhanced (CyaA-KK-AC^-) or strongly reduced (CyaA-QR-AC^-) by specific residue substitutions in the pore-forming domain (Figure 2a). These toxoids were purified close to homogeneity and all contained <120 EU of endotoxin per mg of toxoid. As shown in Figure 2b, the CyaA-AC^- toxoid and mutant CyaA-KK-AC^-, exhibiting an enhanced pore-forming activity, promoted importantly faster leakage of K^+ ions from BMDC than the CyaA-QR-AC^- toxoid that exhibits a reduced pore-forming activity. As the resolution of the here used fluorescence probe-based method was insufficient for demonstrating the quantitative differences in the specific membrane-permeabilizing potencies of the CyaA-AC^- and CyaA-KK-AC^- toxoids at shorter time points after addition, this was previously established in planar lipid bilayer measurements and erythrocyte lysis experiments and was found to be in the order of CyaA-KK-$AC^- \gg$ CyaA-AC^-.[18,21] As further shown in Figure 2c, all purified toxoids bound BMDC *in vitro* and competed equally well for CR3 binding with the biotinylated intact CyaA toxin. As then shown in Figure 2d, 300 ng ml^{-1} of the less pore-forming CyaA-QR-AC^- toxoid did not trigger an upregulation of the maturation marker molecules on BMDC. In contrast, the intact CyaA-AC^- toxoid at 300 ng ml^{-1} and the highly cell-permeabilizing CyaA-KK-AC^- toxoid at 300 ng ml^{-1}, or as well as at 100 ng ml^{-1}, triggered significantly enhanced expression of the maturation molecules, such as the MHC class II molecules I-A/I-E, CD80, CD86 and CD40, respectively, which were upregulated to a similar extent then upon BMDC stimulation with 100 ng ml^{-1} of LPS (Figure 2d). At the same time, the viability of BMDC at the end of incubation with LPS or with the CyaA-AC^- and CyaA-QR-AC^- toxoids at 300 ng ml^{-1} remained high (Figure 2e), whereas the increased cytotoxicity of the highly cell-permeabilizing CyaA-KK-AC^- mutant toxoid could be mitigated by decreasing its working concentration to 100 ng ml^{-1}, as documented in Figure 2e.

As production of inflammatory molecules is a hallmark of the DC maturation process,[22] we next used a cytokine array (Supplementary Figure 1) to analyze inflammatory cytokine and chemokine production induced by CyaA-AC^- in BMDC after 24 h of incubation. As shown in Figure 2f, co-incubation with 300 ng ml^{-1} of CyaA-AC^- enhanced severalfold the production of the IL-6 cytokine, of the chemokines KC (IL-8) and LIX, and of the granulocyte-colony stimulating factor, respectively. However, no enhancement of secretion

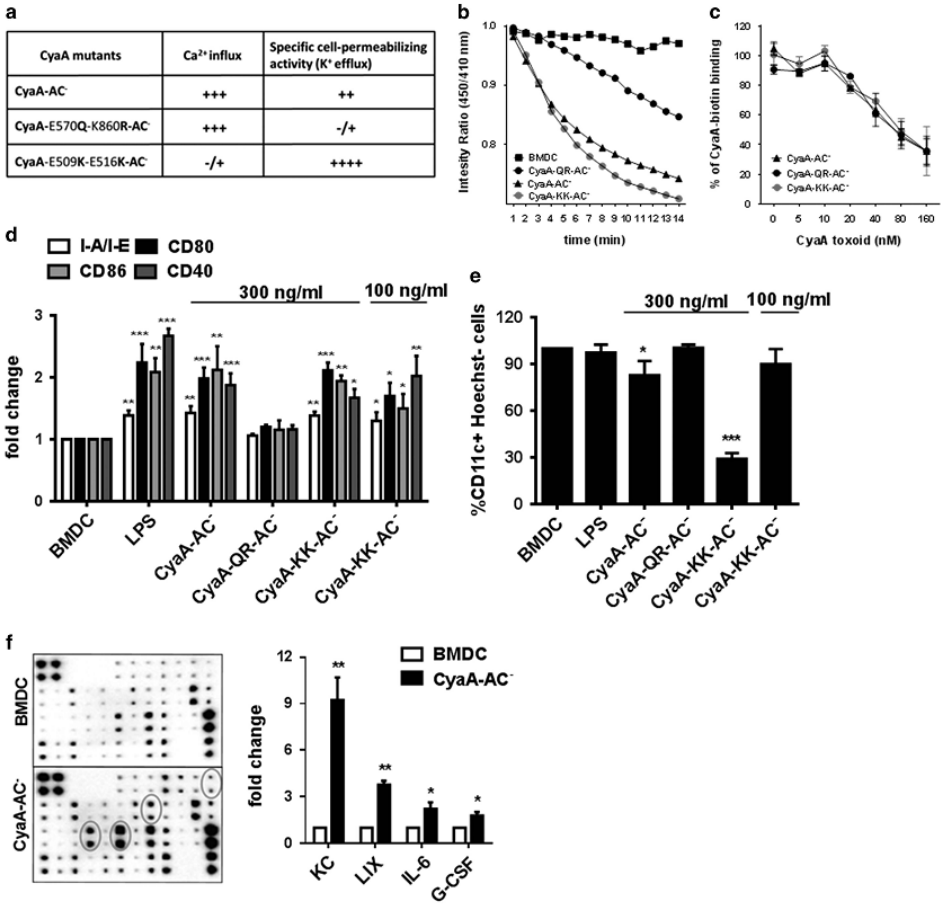

Figure 2 Pore-forming activity-dependent K^+ efflux determines the capacity of CyaA-AC$^-$ toxoid to trigger phenotypic maturation of BMDC. (**a**) CyaA-AC$^-$ toxoids and its mutants used in this study (**b**) Fluorescence measurement of cytosolic K^+ concentration elicited by 3 μg ml^{-1} of indicated toxoids. BMDC were loaded with 9.5 μM PBFI/AM for 30 min at 25 °C in the presence of 0.05% (w/w) Pluronic F-127 in the dark. Fluorescence intensity of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded, ratio of these intensities is shown in the graph. (**c**) Binding of indicated toxoids to BMDC was assessed as a competition for the CD11b/CD18 (CR3) receptor with biotinylated intact CyaA (CyaA-AC$^-$-biotin) and detected with streptavidin-PE by flow cytometry. Graph shows a percentage of CyaA-AC$^-$-biotin bound to BMDC in the presence of non-biotinylated CyaA-AC$^-$, CyaA-QR-AC$^-$ or CyaA-KK-AC$^-$ toxoids. (**d**) BMDC (1×10^6 ml^{-1}) were left untreated, or were incubated with LPS (100 ng ml^{-1}) or with CyaA-AC$^-$, mutant toxoid with reduced pore-forming activity CyaA-QR-AC$^-$ or toxoid with enhanced pore-forming activity CyaA-KK-AC$^-$ at indicated concentrations for 24 h. The expression of I-A/I-E, CD80, CD86 and CD40 was detected in CD11c$^+$Hoechst$^-$ cells by flow cytometry. Graphs represent the means ± s.e. from $n=4$ expressed as fold change of marker expression on mock-treated BMDC (*$P<0.05$, **$P<0.005$ and ***$P<0.001$). (**e**) Cell viability was determined as % of CD11c$^+$ Hoechst$^-$ cells. (**f**) BMDC (1×10^6 ml^{-1}) were left untreated, or incubated with CyaA-AC$^-$ (300 ng ml^{-1}) for 24 h. The inflammatory cytokines and chemokines were evaluated from cell culture supernatant using cytokine array kit with chemiluminescence detection. Membranes are representative of $n=3$. Graph represent fold change of chemiluminiscent signal for each cytokine obtained in mock-treated BMDC (*$P<0.05$ *and* **$P<0.005$). A full colour version of this figure is available at the *Immunology and Cell Biology* journal online.

of the main pro-inflammatory cytokines tumor-necrosis factor-α, IL-12 and IL-1β was observed. These results confirm that the used CyaA-AC$^-$ toxoid preparations were free of detectable TLR ligand contamination and that already at 300 ng ml^{-1} the CyaA-AC$^-$ toxoid-induced phenotypic maturation BMDC through its pore-forming activity that causes K^+ efflux form cells.

Pore-forming activity of CyaA-AC$^-$ toxoid drives maturation marker expression in BMDC via activation of p38 and JNK MAPKs

Potassium efflux from cells can trigger MAPK activation[23] and expression of DC maturation markers was shown to be regulated by signaling involving MAPKs such as p38, JNK and ERK and NF-κB translocation into cell nucleus.[22,24–27] Therefore, 1 h before addition of

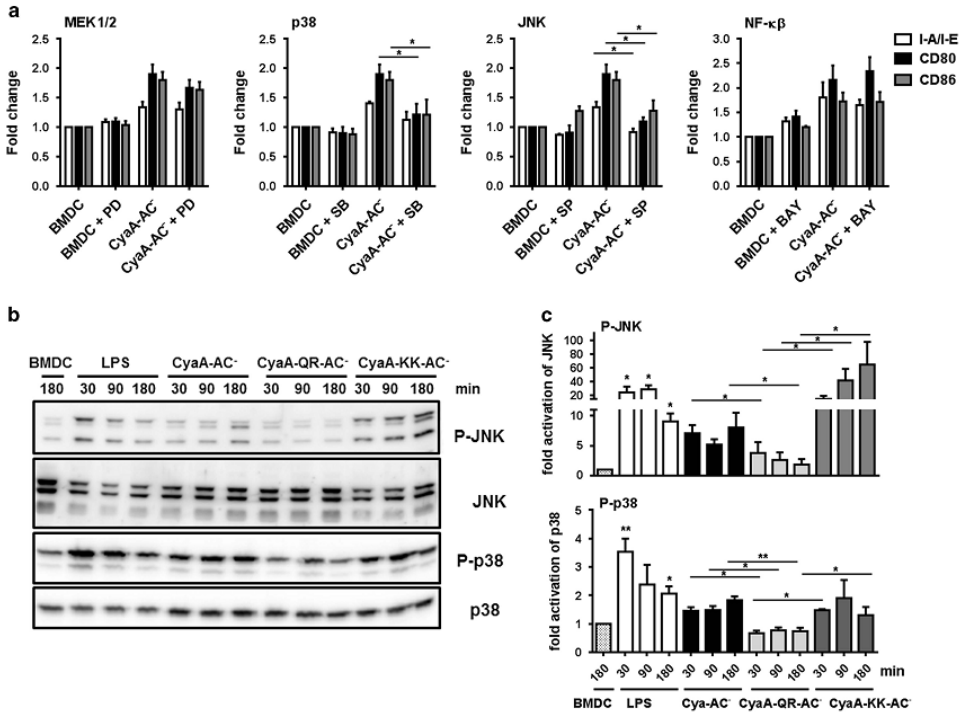

Figure 3 Pore-forming activity-dependent K^+ efflux induced by CyaA-AC$^-$ toxoid drives maturation marker expression in BMDC via p38 and JNK MAPK activation. (**a**) BMDC (1x10^6 per ml) were pre-incubated with the MEK1/2 (ERK1/2) inhibitor PD98059 (PD) p38 inhibitor SB203580 (SB), JNK inhibitor SP600125 (SP) or NF-κβ inhibitor BAY-11-7082 (BAY) all at 10 μM for 1 h before CyaA-AC$^-$ toxoid (300 ng ml^{-1}) was added. After 24 h of incubation the expression of I-A/I-E, CD80 and CD86 molecules on CD11c$^+$Hoechst$^-$ cells was determined by flow cytometry. Values represent the means ± s.e. from $n=3$ expressed as fold change of marker expression on mock-treated BMDC (*$P<0.05$). (**b**) BMDC (1×10^6 ml^{-1}) were incubated with LPS (100 ng ml^{-1}) or indicated CyaA-AC$^-$ mutants (300 ng ml^{-1}) for indicated time and the amount of phosphorylated and total MAPKs were detected by specific antibodies using Western blot analysis. The immunoblots are representative of $n=3$. (**c**) Relative JNK and p38 activation was quantified as P-JNK and P-p38 signal intensities normalized to the respective total JNK and p38 signal. Ratio of signals in lyzates of mock-treated BMDC at time point 180 min was set as 1. The signal intensity data show means ± s.e. from $n=3$ (*$P<0.05$, **$P<0.005$).

the CyaA-AC$^-$ toxoid (300 ng ml^{-1}), the BMDC were pre-incubated with 10 μM MAPK inhibitors, such as the MEK1/2 (ERK1/2) inhibitor PD98059 (PD), the p38 inhibitor SB203580 (SB), the JNK inhibitor SP600125 (SP) or the NF-κβ inhibitor BAY-11-7082 (BAY), respectively. After 24 h of incubation with the toxoid, the expression of I-A/I-E, CD80 and CD86 molecules on CD11c$^+$Hoechst-negative cells was determined by flow cytometry. As shown in Figures 3a, a significant decrease of CyaA-AC$^-$ induced expression of I-A/I-E, CD80 and CD86 molecules was observed upon inhibition of p38 and JNK signaling, even if expression of I-A/I-E was not statistically different in the presence of the p38 inhibitor. The enhancement of expression of I-A/I-E, CD80 and CD86 molecules by CyaA-AC$^-$ treatment was, however, not affected in BMDC treated with MEK1/2 (ERK1/2) and NF-κβ inhibitors.

To investigate whether the pore-forming activity of CyaA-AC$^-$ (K^+ efflux) accounted for the activation of p38 and JNK signaling in BMDC, cells were incubated with LPS or CyaA-AC$^-$ and its mutant variants CyaA-QR-AC$^-$ and CyaA-KK-AC$^-$, exhibiting reduced or enhanced pore-forming activity, respectively. The amounts of phosphorylated and total JNK and p38 kinases were detected by immunoblots after 30, 90 or 180 min of cell exposure to toxoids (Figure 3b). Quantification of the signals (Figure 3c) of phospo-MAPK-specific antibodies then revealed that both JNK and p38 phosphorylation was activated above the background levels only by the pore-forming CyaA-AC$^-$ and CyaA-KK-AC$^-$ toxoids that were capable to provoke K^+ efflux from cells, but not by the CyaA-QR-AC$^-$ toxoid. These findings suggest that the cell-permeabilizing activity and K^+ efflux induced by CyaA-AC$^-$ activated JNK and p38 MAPK signaling and this mediated expression of co-stimulatory molecules on BMDC.

Pore-forming activity of CyaA-AC$^-$ promotes chemotactic migration of BMDC through p38 and JNK MAPKs activation

Activated DC migrate *in vivo* from the periphery into the lymphoid tissue.[28] This involves enhanced expression of the CCR7 receptor of CCL19 and CCL21 chemokines[29] and enhanced production of prostaglandin E2 (PGE2) that contributes to activation of the CCR7 receptor.[30] We thus analyzed if activation by CyaA-AC$^-$ toxoid (300 ng ml^{-1}) also enhanced the migratory capacity of BMDC.

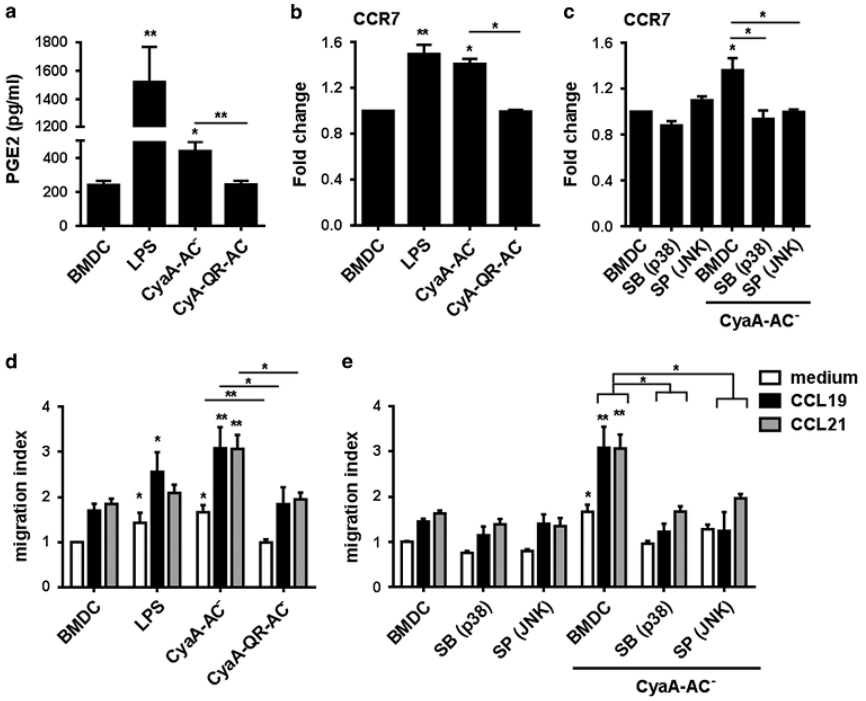

Figure 4 Pore-forming activity of CyaA-AC$^-$ promotes chemotactic migration of BMDC via JNK and p38 activation. BMDC ($1x10^6$ per ml) were incubated with LPS (100 ng ml^{-1}) or CyaA-AC$^-$ or CyaA-QR-AC$^-$ mutant (300 ng ml^{-1}) for 24 h (**a**, **b** and **d**) or pre-incubated with the p38 inhibitor SB203580 (SB; 10 μM) or JNK inhibitor SP600125 (SP; 10 μM) for 1 h before the addition of toxoids (**c** and **e**). (**a**) PGE2 production in BMDC culture supernatants was determined by enzyme-linked immunosorbent assay after 24 h. Data represent means ± s.e. of $n=3$. (**b** and **c**) The expression of CCR7 detected by flow cytometry in CD11c$^+$Hoechst$^-$ cells after 24 h. Values represent the means ± S.E from $n=4$ expressed as fold change of CCR7 expression on mock-treated BMDC (*$P<0.05$, **$P<0.005$). (**d** and **e**) After 24 h of toxoids treatments, cells were washed and allowed to migrate for 4 h across Transwell membrane towards medium alone or containing CCL19 or CCL21 (200 ng ml^{-1}) chemokines. Transmigrated cells (Hoechst$^+$) were counted by flow cytometry. Values represent the means ± s.e. of $n=3$. The number of transmigrated mock-treated BMDC was set as 1 (migration index) (*$P<0.05$, **$P<0.005$).

As shown in Figure 4a, enhancement of production of PGE2 was observed with BMDC exposed to the pore-forming CyaA-AC$^-$ but not on treatment with low-permeabilizing CyaA-QR-AC$^-$ toxoid. The same pattern was observed also for CCR7 expression (Figure 4b). This could then be inhibited by pre-treatment of BMDC with JNK and p38 inhibitors before CyaA-AC$^-$ addition (Figure 4c). Finally, as shown in Figure 4d, activation of BMDC for 24 h by 300 ng ml^{-1} of CyaA-AC$^-$, but not by CyaA-QR-AC$^-$, enhanced significantly the capacity of BMDC to migrate from the upper chamber across the transwell plate membrane into the lower chamber medium that contained chemokines CCL19 or CCL21. As documented in Figure 4e, the p38 and JNK inhibitors interfered with CyaA-AC$^-$ toxoid-induced migration toward both cytokines. Hence, cell permeabilization by CyaA-AC$^-$ toxoid enhanced the migratory capacity of BMDC by a mechanism involving p38 and JNK signaling.

Pore-forming capacity of CyaA-AC$^-$ toxoid induces maturation of splenic DC and expansion of antigen-specific CD8$^+$ and CD4$^+$ T cells *in vivo*

To corroborate that the CyaA-AC$^-$ toxoid can promote maturation of DC also *in vivo*, 25 μg of the CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoids were i.v. injected into mouse tail vein in PBS with 1M urea and the same buffer was used in control mice. Splenocytes were isolated 24 h later and the expression of maturation markers I-A/I-E, CD80, CD86 and CD40 was assessed by flow cytometry, splitting the lymphoid-tissue resident CD11c$^+$ DC by gating into the CD8$^+$CD11b$^-$ and CD8$^-$ CD11b$^+$ subpopulations.[31] As documented by representative dot plots in Figure 5a, both toxoids similarly decreased the percentage of CD8$^-$ CD11b$^+$ cells by 13 − 15%, as compared with control mock-treated mice. This likely reflected the internalization of the CD11b receptor resulting from toxoid binding. However, the percentage of CD8$^+$ CD11b$^-$ cells remained similar among all experimental groups. Quantification of I-A/I-E, CD80, CD86 and CD40 expression in both splenic DC subpopulations is shown in Figure 5b. The expression of CD80, CD86 and CD40, but not of I-A/I-E molecules, was significantly enhanced on CD8$^-$CD11b$^+$ DC from mice injected with CyaA-AC$^-$ and CD86 and also to a lower extent on cells from mice that received the CyaA-QR-AC$^-$ toxoid, but not on cells from control mice that received only buffer. Moreover, a significant increase of only CD86, but not of the other markers was observed on CD8$^+$CD11b$^-$ DC lacking the CR3 receptor of CyaA. This shows that as expected, the CyaA-AC$^-$ and CyaA-QR-AC$^-$ toxoids acted predominantly on

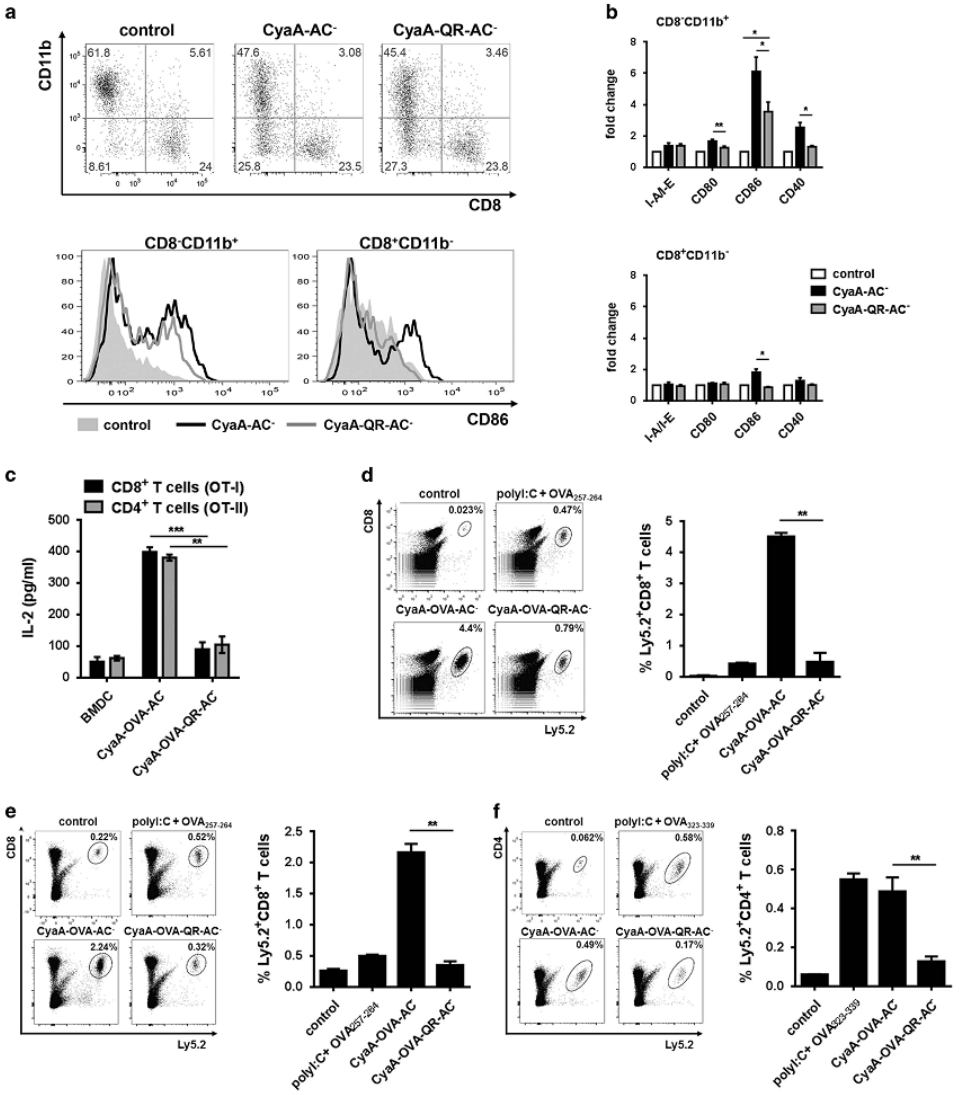

Figure 5 Pore-forming capacity of CyaA-AC$^-$ toxoid induces maturation of splenic DC and expansion of antigen-specific CD8$^+$ T cells *in vivo*. Mice received i.v. PBS with 1 M urea (control) or 25 μg of CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoid. After 24 h mice were killed, total splenocytes were isolated and expression of splenic DC maturation markers was determined by flow cytometry in CD8$^-$CD11b$^+$ and CD8$^+$CD11b$^-$ subpopulations of CD3$^-$CD11c$^+$Hoechst$^-$ cells. (**a**) Dot plots and histograms are representative of $n=3$, 3 mice per group. (**b**) Graphs represent means ± s.e. from $n=3$ expressed as marker fold change of control CD8$^-$CD11b$^+$ DC or CD8$^+$CD11b$^-$ DC (*$P<0.05$, **$P<0.005$). (**c**) BMDC (5×10^4 per sample) were incubated with CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ mutant (300 ng ml^{-1}) for 24 h before washing and co-cultivation with naïve OT-I CD8$^+$ T or OT-II CD4$^+$ T cells (1×10^5 per sample). IL-2 production was determined from cell culture supernatants by enzyme-linked immunosorbent assay after 3 days. Graph represents means ± s.e. from $n=3$. (**d**) Expansion of adoptively transferred CD8$^+$ T cells by BMDC pre-treated 24 h with CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ (300 ng ml^{-1}) *in vivo* was determined after 3 days by flow cytometry as a percentage of CD8$^+$Ly5.2$^+$ T cells out of 2×10^6 counted spleen cells. Dot plots are representative of $n=2$, 2 mice per group. Graphs represent means ± s.e. (**e** and **f**) Expansion of adoptively transferred OVA-specific CD8$^+$ (**e**) and CD4$^+$ (**f**) T cells by i.v. administered CyaA-OVA-AC$^-$, or CyaA-OVA-QR-AC$^-$ (25 μg ml^{-1}), was determined after 4 days by flow cytometry as a percentage of CD8$^+$Ly5.2$^+$ or CD4$^+$Ly5.2$^+$ T cells, respectively, using 2×10^6 counted spleen cells. Dot plots are representative of $n=2$, 3 mice per group. Graphs represent means ± s.e. A full colour version of this figure is available at the *Immunology and Cell Biology* journal online.

$CD11c^+CD8^-CD11b^+$ DC *in vivo*, with a potency reflecting their respective cell-permeabilizing capacities.

Therefore, we investigated if DC maturation induced by pore-forming activity of CyaA-AC^- impacted on T-cell stimulation *in vitro* and *in vivo*. BMDC were incubated with CyaA-OVA-AC^- or the non-permeabilizing CyaA-OVA-QR-AC^- toxoid for 24 h. The cells were then washed and OVA-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells were added for 3 days. As shown in Figure 5c BMDC treated with CyaA-OVA-AC^- stimulated higher IL-2 production in both $CD8^+$ and $CD4^+$ T cells than BMDC incubated with low pore-forming mutant CyaA-OVA-QR-AC^-. Similarly, as shown in Figure 5d, in contrast to BMDC treated with low-permeabilizing CyaA-OVA-QR-AC^- toxoid, the BMDC treated *ex vivo* with CyaA-OVA-AC^- for 24 h and subsequently injected into mice were capable of expanding adoptively transferred $CD8^+$ T cells. These data suggest that pore-forming activity of CyaA-AC^- endowed DC with a higher T-cell stimulatory capacity *in vitro* and *in vivo*. Finally, we tested if this activity translated directly into a higher capacity of CyaA-OVA-AC^- to induce antigen-specific $CD8^+$ and $CD4^+$ T-cell responses *in vivo* at a dose of 25 μg of the toxoid. This reduced dose was used here because at the previously used higher dose of 50 μg of the same toxoids, no difference in T-cell inducing potency of the two toxoids was observed in an *in vivo* killing assay, likely due to saturation of the response. Here CyaA-OVA-AC^- or CyaA-OVA-QR-AC^- were injected at 25 μg i.v. into mice 1 day after the adoptive transfer of OVA-specific $CD8^+$ or $CD4^+$ T cells and T-cell expansion was detected 4 days later by flow cytometry. As shown in Figures 5e and f, CyaA-OVA-AC^- induced significantly higher expansion of OVA-specific $CD8^+$ and $CD4^+$ T cells.

DISCUSSION

In this study, we identified a novel intrinsic adjuvant activity of CyaA-AC^-. We show that non-enzymatic but pore-forming CyaA-AC^- toxoid at as low concentration as only 300 ng ml^{-1} induces maturation of CD11b-expressing DC by a mechanism that is independent of TLR 2, 4 and 9, CD14, TRIF, MyD88 or inflammasome signaling. The CyaA-AC^--induced DC maturation depended on the specific pore-forming capacity of the toxoid that permeabilized cells and induced K^+ efflux that triggered activation of the JNK and p38 MAPKs. We further show that the adjuvanting pore-forming activity of CyaA-AC^- on DC contributes to the potency of the toxoid in induction of T-cell responses both *in vitro* and *in vivo*.

We observed that as low amounts as 300 ng ml^{-1} of CyaA-AC^- carrying antigenic ovalbumin T-cell epitopes endowed BMDC with a capacity to effectively stimulate $CD8^+$ and $CD4^+$ T-cell responses *in vitro*. At this toxoid concentration, the CyaA-AC^- induced phenotypic maturation of BMDC led to production of KC (IL-8) and IL-6 cytokines, of the chemokine LIX and of the differentiation factor granulocyte-colony stimulating factor, respectively. Interestingly, all of these molecules regulate predominantly the activities of neutrophils that are important mediators of innate immunity. We have not detected production of the major pro-inflammatory cytokines such as tumor-necrosis factor-α, IL-12p70 or IL-1β by toxoid-activated BMDC, which reflects the absence of TLR signaling of CyaA-AC^- at the low toxoid concentrations. This was also confirmed by the capacity of the CyaA-AC^- toxoid to trigger CD80 and CD86 expression in BMDC generated from TLR 2, 4, 2/4 and 9, TRIF, MyD88 and CD14 knockout mice. These experiments clearly showed that if traces of TLR ligands were still present in the used toxoid preparations, their levels were too low to activate the expression of co-stimulatory molecules in BMDC any importantly. Similarly, the expression of CD80 and CD86 induced in BMDC generated from mice deficient in inflammasome signaling proteins, such as IL-1 R, P2X7, ASC and NLRP3, was not decreased. This shows that whereas CyaA pore formation-dependent K^+ efflux activates NLRP3 complex formation and stimulates IL-1β production under infection with *B. pertussis*, there is no inflammasome activation by enzymatically inactive CyaA-AC^- alone in the absence of BMDC priming through TLR signaling.[9] Similarly, there was no involvement of inflammasome signaling in the $CD8^+$ T-cell stimulatory adjuvant activity of CyaA-AC^- *in vivo*, as shown in the recent study of Dadaglio *et al.*[17] We identified here that K^+ efflux induced by CyaA-AC^--mediated cell permeabilization acts as a major trigger for CyaA-AC^--induced BMDC maturation and migration. This goes well with the published effects of K^+ and Ca^{2+} ion signaling on induction of DC maturation and migration towards CCL21.[20,32] Of note, Ca^{2+} influx, which accompanies the translocation of AC domain into the cells,[5–7] must not necessarily have a significant role in CyaA-AC^--induced BMDC maturation. Indeed, the highly pore-forming toxoid CyaA-KK-AC^- triggers very little Ca^{2+} influx,[5] while induced efficient BMDC maturation. Conversely, the low pore-forming mutant CyaA-QR-AC^- did not stimulate BMDC's maturation and migration, despite inducing Ca^{2+} influx into the cells to a similar extent as the wild type toxoid CyaA-AC^-.[6] Nevertheless, some cooperation between Ca^{2+} and K^+ signaling in CyaA-AC^--mediated DC maturation cannot be fully excluded at present.

The activation of p38 and JNK signaling pathways by CyaA-AC^- is in agreement with the role of stress-activated p38 and JNK MAPKs in numerous processes underlying induction of adaptive immune responses, including DC maturation and chemotactic migration.[33] Whereas *in vitro* CD80 and CD86 molecules seem to be regulated by CyaA-AC^- pore-dependent activation of both kinases, the expression of I-A/I-E may be predominantly regulated only by the JNK kinase. This would go well with a described differential regulation of DC maturation markers[34,35] but it would not explain the lack of I-A/I-E expression on splenic DC that is observed after *in vivo* toxoid administration. Hence, a more complex regulation of DC maturation may occur under *in vivo* conditions. In our study, p38 and JNK activation was also involved in upregulation of CCR7 and enhancement of chemotactic migratory capacity of the maturing BMDC towards CCL19 and CCL21, as inhibition of these signaling pathways abolished CyaA-AC^--mediated induction of BMDC migration. This is in agreement with previous reports that p38 and JNK kinases are involved in DC migration.[28,36,37] p38 activity triggers synthesis and secretion of PGE2[38] and this might explain the observed enhancement in PGE2 production induced by CyaA-AC^- in BMDC. Interestingly, K^+ efflux from cells permeabilized by pore-forming toxins was recently shown to trigger specifically the phosphorylation of p38 (ref. 23) and JNK kinases.[39] This appears to reflect a highly conserved mechanism of cellular stress response to pore-forming toxins attack on cell membrane integrity, which is found in nematodes, insects and mammals.[40–42]

Over the past 20 years, various CyaA toxoids were used without any added adjuvant to successfully deliver antigens into cytosol of dendritic cells for induction of potent protective anti-viral and anti-tumor $CD8^+$ T-cell-mediated immunity in mice.[13] It is, therefore, plausible to propose that it is the self-adjuvanting capacity of the CyaA-AC^- toxoid, that is, its capacity to trigger maturation and migration of DC, which accounts for its potent T-cell stimulatory capacity *in vivo*. We show here that the i.v. administered CyaA-AC^- toxoid (25 μg) strongly enhances CD86 expression and upregulates CD80 and CD40 molecules on splenic $CD11c^+CD8^-CD11b^+$ DC *in vivo*. Intriguingly, at a dose of 25 μg per mice, the CyaA-QR-AC^- toxoid was still capable to

rather efficiently trigger a lower but still significant expression of CD86 on splenic $CD11c^+CD8^+CD11b^-$ DC, despite its reduced cell-permeabilizing activity (c.f. Figure 5). We cannot exclude that at the high CyaA-QR-AC^- concentration its residual pore-forming activity may still promote enough K^+ efflux from DC to prime CD86 expression *in vivo*.

Recently, Dadaglio *et al.* showed that after the intravenous application of a higher dose of 50 μg of LPS-free CyaA-Tyr toxoid the permeabilization-primed maturation of splenic DC is largely potentiated by signaling through the TLR4/TRIF pathway.[17] This signaling may result from a direct interaction/clustering of toxoid-CD11b/CD18 complexes with TLR4 receptors in the membranes of $CD11b^+$ DC. In keeping with these results, we show here that CyaA-AC^- at concentrations 0.1–<1 μg ml^{-1} induces DC maturation solely via potassium efflux, whereas at concentration ⩾1 μg ml^{-1}, the CyaA-AC^- induces DC maturation through both the potassium efflux-triggered and the TLR4-dependent mechanisms, presumably operating in synergy. This is most likely potentiating the induction of antigen-specific T-cell responses by CyaA toxoids. Indeed, the induction of antigen-specific $CD8^+$ T-cell response after administration of CyaA-AC^- carrying ovalbumin epitope SIINFEKL was shown to depend only in part of the TLR4/TRIF signaling triggered by the toxoid.[17] This is explained by our present observation that the pore-forming activity of CyaA-AC^- is necessary for toxoid-treated BMDC to induce T-cell responses. We show here, indeed, that BMDC treated with low concentration of wild type CyaA-OVA-AC^- (300 ng ml^{-1}), but not with the less pore-forming toxoid CyaA-OVA-QR-AC^-, were able to induce $CD8^+$ and $CD4^+$ T-cell responses *in vitro* and expanded adoptively transferred $CD8^+$ T cells *in vivo*. Moreover, we observed a significantly higher expansion of adoptively transferred OVA-specific $CD8^+$ and $CD4^+$ T cells after administration of 25 μg ml^{-1} of CyaA-OVA-AC^- in comparison to administration of CyaA-OVA-QR-AC^-. This confirms the higher stimulatory activity of the pore-forming wild type toxoid towards antigen presenting cells *in vivo*. Based on these findings, we propose a concentration-dependent model of adjuvant activity of the immunotherapeutically relevant CyaA-AC^- toxoid (Figure 6). It predicts that at low toxoid concentrations the cell-permeabilizing activity provokes K^+-efflux-mediated p38 and JNK activation, triggering DC maturation, which is largely potentiated by TLR4/TRIF signaling upon high level of toxoid binding to cells.

In conclusion, the present study demonstrates that on top of the previously described ability to deliver passenger antigens into cytosol of DC for processing and subsequent presentation on surface MHC molecules to T cells, the capacity of the CyaA-AC^- toxoid to permeabilize DC triggers their maturation. This is then required for efficient antigen-specific priming of T cells to induce anti-tumor or anti-viral immunity.[13] The CyaA-AC^- toxoid was further shown to induce a prominently Th1-polarized type of immune responses.[43,44] This observation is potentially relevant also for the use of the toxoid as a new antigen and adjuvant in the next generation of acellular pertussis vaccines. Indeed, shifting of the predominantly Th2-polarized immune response to currently used acellular pertussis vaccines towards induction of a more Th1/Th17-polarized responses appears to be highly desirable in view of induction of longer lasting and more efficient protective immunity against *B. pertussis* infection.[45,46]

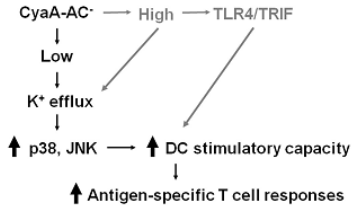

Figure 6 Proposed mechanism of adjuvanticity of CyaA-AC^-. A model of CyaA-AC^--mediated enhancement of T-cell stimulatory functions of DC which depends on pore-forming-dependent K^+ efflux at low toxoid concentrations and it is potentiated by TLR4/TRIF signaling[17] at higher toxoid concentrations. Gray untested hypotheses in this study.

METHODS

Production and purification of CyaA-AC^- and its mutants

The CyaA-AC^- toxoid is devoid of adenylate cyclase enzyme activity due to insertion of the GlySer dipeptide between residues 188 and 189 in the ATP-binding site of the AC domain.[47] The toxoid variants used in this study were CyaA-E570Q-K860R-AC^- (abbreviated CyaA-QR-AC^-), a mutant with strongly reduced pore-forming activity and CyaA-E509K-E516K-AC^- (abbreviated CyaA-KK-AC^-) a mutant with an increased pore-forming activity.[5,18,19,21] CyaA-AC^- and low-permeabilizing CyaA-QR-AC^- toxoids carrying MHC class I epitope from chicken egg ovalbumin SIINFEKL (CyaA-SIINFEKL-AC^-) inserted in position of 233 of AC domain are described in.[17,19] In this study, we constructed a new CyaA-OVA-AC^- toxoid and low-permeabilizing CyaA-OVA-QR-AC^- which carry a polyepitope consisting of three chicken egg ovalbumin epitopes: $OVA_{257–264}$ (SIINFEKL) for MHC class I and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) and $OVA_{258–276}$ (IINFEKLTEWTSSNVMEER) for MHC class II presentation (CyaA-OVA-AC^-), respectively, inserted at position 233 of the AC domain. CyaA-AC^- derived toxoids were produced in *E. coli* BL21/pMM100(*lacI*^{q}) and were purified close to homogeneity as described previously.[48,49] The endotoxin content in all CyaA samples was determined by the Limulus amebocyte lysate assay (QCL-1000; Cambrex, East Rutherford, NJ, USA) according to the manufacturer's instructions and was below 120 EU mg^{-1}.

Cell binding of CyaA-AC^- and its mutants

The competitive CyaA binding assay was performed as described elsewhere[50,51]. Briefly, BMDC (5×0^5 per well) in 100 μl of DMEM with 1% FCS were preincubated with different concentrations of various toxoids for 30 min on ice. Then, the competitor CyaA-biotin (30 nM) was added for another 30 min. Finally, unbound proteins were removed by repeated washing, and cells were stained with PE-conjugated streptavidin (Exbio, Prague, Czech Republic) at 1:400 dilution (50 μl per well) for 30 min on ice. The amount of surface bound CyaA-biotin was then determined by flow cytometry using a FACS LSR II instrument (BD Biosciences, San Jose, CA, USA) and FlowJo version 7.2.1 (Treestar, Inc., Ashland, OR, USA). Results are expressed as the percent of CyaA-biotin binding, which means: CyaA-biotin bound in the presence of the competitor/maximal CyaA-biotin binding in the absence of the competitor CyaA x100 (%).

Potassium efflux

Fluorescence measurement of cytosolic K^+ was performed as described previously.[19] Briefly, BMDC were loaded with 9.5 μM PBFI/AM (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 25 °C in the presence of 0,05% (w/w) Pluronic F-127 (Sigma-Aldrich, Dorset, UK) in the dark. Fluorescence intensity of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded, ratio of these intensities are shown in the graphs.

Mice

Six to twelve-week-old C57BL/6 (Ly5.2) mice were obtained from a breeding colony at the Institute of Physiology of ASCR in Prague, Czech Republic. C57BL/6 (Ly5.1) and OT-I mice were provided by Marek Kovar, Institute of Microbiology of ASCR v.v.i. Prague, Czech Republic. OT-II mice were generous gift of Pavel Otahal, Institute of Molecular Genetics of ASCR, Prague, Czech

Republic. Mice deficient in P2X7,[52] ASC[53] and NLRP3[54] were generous gifts from Prof. Stefan F. Martin, University Medical Center, Freiburg, Germany. Mice deficient for TLR2,[55] TLR4, TLR2/4,[56] TLR9,[57] TRIF,[58] MyD88,[59] CD14,[60] IL-1 R,[61] P2X7, ASC and NLRP3[62] and their corresponding WT C57BL/6 and C57BL/10 progenitor mice were bred under specific pathogen-free conditions in the animal facilities of the Max Planck Institute of Immunobiology and Epigenetics and the University Medical Center, Freiburg, Germany where all of the experimental procedures were performed in accordance with institutional, state and federal guidelines on animal welfare and every effort was made to minimize suffering. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Freiburg Medical Center or the MPI. Mice were anesthetized before sacrificing with 1% pelltobarbitalum natricum at the dose of 10 mg kg^{-1}. All animal experiments were also approved by the competent Animal Welfare Committee of the Institute of Microbiology of the ASCR v.v.i. and handling of animals was performed according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National Assembly, Collection of Laws No. 149/2004, inclusive of the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws No. 207/2004, on care and use of experimental animals.

Generation of bone marrow-derived dendritic cells

BMDC were generated according to the method of Lutz[63]. Bones for isolation of BMDC of knockout and corresponding wild type mice were prepared at the Max Planck Institute of Immunobiology and Epigenetics and the University of Freiburg Medical Center and shipped on ice. Briefly, bone marrow cells were flushed from femurs and tibias of mice, and cultured at 2×10^6 ml^{-1} in 100 mm dishes in 10 ml of RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mg-ml^{-1} streptomycin, 1000 U ml^{-1} penicillin and 0.25 µg ml^{-1} amphotericin (Sigma-Aldrich), 50 µ 2-mercaptoethanol, 1% non-essential amino acids (Biochrom), 1 mM sodium pyruvate, 2 mM glutamine and 200 U ml^{-1} granulocyte-macrophage colony-stimulating factor. Fresh medium was added on day 3 or changed on day 6. Loosely attached cells were used for experiments at days 6–8. Cultured cells (70–80%) expressed CD11c (APC-anti-mouse-CD11c antibody, clone N418, eBioscience, San Diego, CA, USA) and 90% CD11b (PE-anti-mouse-CD11b antibody, clone M1/70, BD Pharmingen, BD Biosciences).[2] Before all experiments with CyaA toxoids the RPMI medium used for cultivation of BMDC was replaced by DMEM medium to avoid uncontrollable chelation of calcium ions by the phosphate ions contained in RPMI medium, as calcium is required for the activity of CyaA. BMDC were allowed to rest in DMEM at 37 °C in 5% CO_2 atmosphere for at least 1 h before performing experiments.

Antibodies and reagents

LPS from *E. coli* serotype 0111:B4, MAPK inhibitors MEK1/2 (ERK1/2) (PD98059), p38 (SB203580) or JNK (SP600125) and NF-κβ inhibitor BAY-11-7082 were obtained from Sigma-Aldrich. The following antibodies were used: APC-anti-mouse CD11c (clone N418), FITC-anti-mouse-I-A/I-E (clone M5/114.15.2), FITC-anti-mouse-CD80 (clone 16-10A1), FITC-anti-mouse-CD86 (clone GL1), PE-anti-mouse CCR7 (CD197; clone 4B12), PE-anti-mouse-CD3 (clone 145-2c11), PE-Cy7-anti-mouse-CD11b (clone M1/70) and PerCP-Cy5.5-anti-mouse-CD8a (53-6.7) from eBioscience or FITC-anti-mouse-CD40 (clone 3.23) and Alexa700-anti-mouse-CD8 (53-6.7), PerCP-anti-mouse-CD4 (RM4-5) and APC-anti-mouse-Ly5.2 (CD45.2 clone 104) from BD Pharmingen. Phospho-p38 (28B10), p38, phospho-SAPK/JNK (G9) and SAPK/JNK antibodies were obtained from Cell Signaling Technology.

Detection of BMDC's viability

BMDC (1×10^6 ml^{-1}) in DMEM at 37 °C in 5% CO_2 atmosphere were left untreated or were treated with LPS (100 ng ml^{-1}) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ (300 ng ml^{-1}) or CyaA-KK-AC (300 or 100 ng ml^{-}), respectively. After 24 h of incubation, the viability of BMDC was detected with Hoechst 33258 (0.5 µg ml^{-1}; Invitrogen, Thermo Fisher Scientific) by flow cytometry as a percentage of CD11c$^+$Hoechst 33258-negative cells.

BMDC's maturation markers and CCR7 expression

BMDC (1×10^6 ml^{-1}) were left untreated or were treated with LPS (100 ng ml^{-1}) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ (300 ng ml^{-1}) or CyaA-KK-AC$^-$ (100 ng ml^{-1}) for 24 h in DMEM at 37 °C in 5% CO_2 atmosphere. In some experiments BMDC were pre-treated with MAPK and NF-κβ (10 µM) inhibitors for 1 h before addition of toxoids. After 24 h the expression of I-A/I-E, CD80, CD86, CD40 and CCR7 on CD11c $^+$Hoechst33258-negative cells was detected by flow cytometry. Mean fluorescence intensity values of samples were normalized as fold change of marker expression of untreated control BMDC.

Cytokine and prostaglandin E2 production

For inflammatory cytokine and chemokine detection BMDC (1×10^6 ml^{-1}) were left untreated or were incubated with CyaA-AC$^-$ at 300 ng ml^{-1}. After 24 h the level of cytokines and chemokines in culture supernatants was assessed by mouse inflammation antibody array kit (RayBiotech, Norcross, GA, USA) according to manufacturer's instructions. The chemiluminescence was detected using LAS-1000 (Luminiscence Analyzing System, Fuji) and analyzed by AIDA 1000/1D Image Analyzer software, version 3.28 (Raytest Isopenmessgeraete GmbH, Straubenhardt, Germany). The final graph shows the ratio of relative production of cytokines and chemokines induced by CyaA-AC$^-$ that were normalized to the background production of untreated control BMDC. For PGE2 detection, the BMDC (1×10^6 ml^{-1}) were left untreated, or were treated with LPS (100 ng ml^{-1}) or CyaA-AC$^-$ or CyaA-QR-AC$^-$ (300 ng ml^{-1}) for 24 h. Culture supernatants were collected and the amount of PGE2 was detected using the PGE2 EIA Kit (Cayman Chemicals, Ann Harbor, MI, USA) according to manufacturer's instructions.

Western blot analysis of p38 and JNK MAPK activation

BMDC (1×10^6 ml^{-1}) were left untreated, or incubated with LPS (100 ng ml^{-1}) or with CyaA-AC$^-$, CyaA-QR-AC$^-$ or CyaA-KK-AC$^-$ for 30, 90 and 180 min. The BMDC were placed on ice and lysed for 10 min with 100-µl lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 50 mM NaF, 10 nM Calyculin A and Complete Mini protease inhibitors (Roche Diagnostics, Basel, Switzerland). The samples were boiled with Laemmli buffer for 5 min and analyzed by western blot using specific antibodies recognizing phosphorylated and total JNK and p38, respectively. The signal of secondary HRP-conjugated antibody (GE Healthcare, Little Chalfont, UK) was detected by the West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific) on LAS-4000 (Luminiscence Analyzing System, Fuji, Edison, NJ, USA) and quantified by AIDA 1000/1D Image Analyzer software (Straubenhardt, Germany) as the ratio of signal of phosphorylated p38/JNK to non-phosphorylated total p38/JNK, respectively.

BMDC migration

BMDC (1×10^6 ml^{-1}) were left untreated, or were incubated with 100 ng ml^{-1} LPS or with CyaA-AC$^-$ or CyaA-QR-AC$^-$ (300 ng ml^{-1}). In some experiments BMDC were pre-treated with MAPK inhibitors (10 µM) for 1 h before addition of toxoids. After 24 h, cells were washed and aliquots of 1×10^5 BMDC were transferred to the upper chambers of 96-well transwell cell culture plates with 5-µm pore size polycarbonate filters (Corning Costar, Tewksburg, MA, USA). The lower chambers of the transwell plates were filled with DMEM medium with or without 200 ng ml^{-1} of chemokine CCL19 or CCL21 (Peprotech, Rocky Hill, NJ, USA). BMDC were allowed to migrate for 4 h and transmigrated cells were subsequently stained for viability by Hoechst 33258 and counted by flow cytometry. The number of transmigrated mock-treated BMDC was set to 1 (migration index).

Splenic DC maturation *in vivo*

C57BL/6 mice were i.v. injected into tail vein with PBS buffer with 1M urea (control), or with 25 µg of CyaA-AC$^-$ or CyaA-QR-AC$^-$ toxoids diluted in PBS with 1M urea. After 24 h mice were sacrificed, the spleens were removed and cut into pieces and dissociated with Colagenase D (1 mg ml^{-1}) for 30 min at 37 °C. Cellular suspensions were filtered through a 70-µm cell strainer and erythrocytes were lysed using ACK Lysing buffer (Life Technologies) for

10 min. Cell suspensions were centrifuged for 5 min at 300 *g* and filtered through a 30 μm strainer. The expression of maturation markers I-A/I-E, CD80, CD86 and CD40 was detected on living (Hoechst 33258-negative) $CD3^-CD11c^+CD8^-CD11b^+$ or $CD3^-CD11c^+CD8^+CD11b^-$ subpopulations of splenic DC by flow cytometry.

T-cell expansion *in vitro* and *in vivo*

For T-cell expansion *in vitro*, the BMDC (5×10^4 per sample) were left untreated or were incubated with CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ (300 ng ml^{-1}) for 24 h, repeatedly washed and co-incubated for 3 days with naïve OVA-specific $CD8^+$ (OT-I) or $CD4^+$ (OT-II) T cells ($2x10^5$ per sample). T cells were isolated from lymph nodes and spleens of OT-I or OT-II transgenic mice by a magnetic cell separation using $CD8^+$ or $CD4^+$ T-cell Isolation kits (Miltenyi, Gladbach, Germany), respectively. IL-2 production was determined from cell culture supernatants by enzyme-linked immunosorbent assay. For T-cell expansion *in vivo*, purified OVA-specific $CD8^+$ or $CD4^+$ T cells (Ly5.2) were injected i.v. into C57BL/6 recipients (Ly5.1) at 1.5×10^6 cells per mouse. After 24 h mice were injected *i.v.* with 1.5×10^6 BMDC that had been pre-treated with 300 ng ml^{-1} of CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ for 24 h or with 25 μg CyaA-OVA-AC$^-$ or CyaA-OVA-QR-AC$^-$ toxoids. As a positive control for T-cell proliferation, mice were injected i.p. with or $OVA_{257–264}$ peptide (SIINFEKL)+polyI:C (75 μg) or $OVA_{323–339}$ peptide (ISQAVHAA-HAEINEAGR)+polyI:C (75 μg), respectively. As a negative control mice were injected with non-treated DC or PBS+urea (2M). Four days later, spleen cells were harvested, fixed by 4% paraformaldehyde and the expansion of adoptively transferred $CD8^+Ly5.2^+$ or $CD4^+Ly5.2^+$ T cells, respectively, was detected by flow cytometry.

Statistical analysis

The significance of differences between groups was determined by the unpaired two-tailed Student's *t*-test and analysis of variance. Experimental data were compared to those obtained with the corresponding controls and differences were considered statistically significant if $P<0.05$ (*), $P<0.005$ (**) or $P<0.001$ (***).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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6.3 PUBLICATION 2

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Adenylate cyclase toxin (CyaA) is a key virulence factor of the whooping cough agent *Bordetella pertussis*. The toxin targets primarily phagocytes possessing the CD11b/CD18 integrin receptor. By delivering an adenylyl cyclase (AC) enzyme into their cytosol the CyaA subverts cellular signaling by elevating cAMP levels. We got interested in what role does CyaA actually play during *B. pertussis* infection, particularly in immunomodulation of DC. CyaA toxin was described to have a potent immunomodulatory activity and it was shown to change the maturation profile of LPS-stimulated DC. We wanted to determine whether it can influence also the migratory capacity of LPS-stimulated DC and how all these CyaA mediated manipulation interferes with the T cell stimulatory capacity of DC. We analysed the modulatory effects of CyaA action on TLR-activated murine and human dendritic cells by characterizing their adhesive, migratory and antigen presenting properties.

We found that cAMP signaling of CyaA enhanced TLR-induced dissolution of cell adhesive contacts and migration of DC towards the lymph node-homing chemokines CCL19 and CCL21 *in vitro*. Using adoptive transfer experiments, we showed that CyaA has the ability to interfere also with the induction of $CD4^+$ and $CD8^+$ T cell responses by TLR-stimulated DC. Upon CyaA treatment, the LPS-activated DC exhibited a decreased capacity to present a protein antigen to $CD4^+$ T cells, while an increased capacity to promote $CD4^+CD25^+Foxp3^+$ T regulatory cells *in vitro* was observed. Furthermore, CyaA also decreased the capacity of LPS-stimulated DC to induce $CD8^+$T cell proliferation. This was accompanied by decreased induction of IFN-γ, while increased IL-17 and IL-10 production by $CD8^+$ T cells was observed. Taken together, our results suggest that via cAMP signaling the CyaA toxin can significantly interfere with the onset of the adaptive immune response

to *B. pertussis* infection, possibly by promoting migration of incompletely matured DC into draining lymph nodes. Such DC are then unable to trigger proper T cell stimulation.

My contribution: Migration experiments with BMDC, generation of BMDC, preparation of some recombinant CyaA toxins/toxoids, helped in T cell expansion and T cell response experiments and some other experiments.

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Bordetella Adenylate Cyclase Toxin Differentially Modulates Toll-Like Receptor-Stimulated Activation, Migration and T Cell Stimulatory Capacity of Dendritic Cells

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Abstract

Adenylate cyclase toxin (CyaA) is a key virulence factor of the whooping cough agent *Bordetella pertussis*. The toxin targets CD11b-expressing phagocytes and delivers into their cytosol an adenylyl cyclase (AC) enzyme that subverts cellular signaling by increasing cAMP levels. In the present study, we analyzed the modulatory effects of CyaA on adhesive, migratory and antigen presenting properties of Toll-like receptor (TLR)-activated murine and human dendritic cells (DCs). cAMP signaling of CyaA enhanced TLR-induced dissolution of cell adhesive contacts and migration of DCs towards the lymph node-homing chemokines CCL19 and CCL21 *in vitro*. Moreover, we examined in detail the capacity of toxin-treated DCs to induce $CD4^+$ and $CD8^+$ T cell responses. Exposure to CyaA decreased the capacity of LPS-stimulated DCs to present soluble protein antigen to $CD4^+$ T cells independently of modulation of co-stimulatory molecules and cytokine production, and enhanced their capacity to promote $CD4^+CD25^+Foxp3^+$ T regulatory cells *in vitro*. In addition, CyaA decreased the capacity of LPS-stimulated DCs to induce $CD8^+$ T cell proliferation and limited the induction of IFN-γ producing $CD8^+$ T cells while enhancing IL-10 and IL-17-production. These results indicate that through activation of cAMP signaling, the CyaA may be mobilizing DCs impaired in T cell stimulatory capacity and arrival of such DCs into draining lymph nodes may than contribute to delay and subversion of host immune responses during *B. pertussis* infection.

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Introduction

Despite extensive vaccination programs, pertussis called also whooping cough, remains the least controlled vaccine-preventable infectious disease and represents a significant health burden worldwide, accounting for as many as 300 000 deaths per year [1]. The currently observed significant upsurge of pertussis incidence in the most developed countries then raises substantial prospective concern about evolution of whooping cough epidemiology [2]. This highly contagious disease is caused by the Gram-negative coccobacilli *Bordetella pertussis* and *B. parapertussis* that adhere to ciliated epithelial cells of human nasopharynx and trachea. The concerted action of virulence factors, such as adhesins and toxins, then enables bacteria to resist the clearance by the mucociliary escalator and host immune system [1,3].

The adenylate cyclase toxin (CyaA) is a key virulence factor of *B. pertussis* that subverts host defense [4]. It has been shown that CyaA-deficient bacteria are unable to cause lethal infection and are cleared rapidly from the lungs in a mouse challenge model [5]. The 1706 aa long protein carries an N-terminal adenylate cyclase (AC) domain (~400 residues) that penetrates into host phagocyte cytosol, eliciting Ca^{2+} influx [6]. Upon binding of intracellular calmodulin, the AC catalyzes conversion of ATP to cAMP, a key second messenger that subverts bactericidal functions of phago-

cytes. The C-terminal RTX hemolysin (Hly) moiety (~1306 residues) then mediates CyaA toxin binding to myeloid phagocytic cells via the $\alpha_M\beta_2$ integrin, known also as CD11b/CD18, complement receptor 3 (CR3), or Mac-1) [7]. The Hly moiety permeablizes target cell membranes by forming cation-selective toxin pores, thus perturbing ion homeostasis [4,8]. CyaA-induced efflux of K^+ ions from the host cell was, indeed, shown to activate the NALP3 inflammasome and promote IL-1β release from LPS-primed dendritic cells (DCs) [9].

CyaA-induced cAMP signaling quickly incapacitates antibacterial functions of macrophages and neutrophils by inhibiting superoxide production, chemotaxis and phagocytosis [10,11] and promotes subsequent apoptosis [12] or necrosis [13]. We observed that by causing transient decrease of RhoA activity, the CyaA provokes subversive membrane ruffling and actin cytoskeleton rearrangements in macrophages, which is accompanied by an immediate shut-down of macropinocytosis [14].

Furthermore, cAMP signaling of CyaA was shown to selectively modulate Toll-like receptor (TLR)-induced activation and maturation of DCs, enhancing IL-10 and inhibiting IL-12p70 production, respectively, and promoting expansion of IL-10-secreting regulatory T cells (Tr1) [15–19]. CyaA activity was further reported to modulate Th1/Th17 polarization induced by *B. pertussis*-treated DCs towards enhanced Th17 and limited Th1 expansion [20]. Th17 and Th1 cells were shown to be involved in clearance of *B. pertussis* from the respiratory tract in mice immunized with a whole cell pertussis vaccine (Pw) [21]. Moreover, IL-1β-induced Th17 cells have been shown to play a critical role in clearance of a primary infection with *B. pertussis* [9]. Although it has been suggested that $CD8^+$ T cells are dispensable for protective immunity to this bacterium [22,23], it has been recently shown that $CD8^+$ T cells participate in the immune response to acute *B. pertussis* infection [24] and pertussis-specific $CD8^+$ memory T cells are induced by vaccination in humans [24,25].

Here, we extended the studies on immunomodulatory action of CyaA on TLR-activated mouse bone-marrow derived DCs (BMDCs) and human monocyte-derived DCs (MDDCs) using a close to physiologically low toxin concentration [26]. We show that CyaA accelerates LPS-induced cell detachment and migration towards the lymph node-homing cytokines CCL19 and CCL21 *in vitro*. Such DCs exhibited a decreased capability to stimulate proliferation of antigen-specific $CD4^+$ and $CD8^+$ T cells *in vitro* and *in vivo*, which was independent of their capacity to engulf and degrade protein antigens. Moreover, CyaA treatment of DCs decreased their ability to induce IFN-γ-secreting $CD8^+$ T cells, but promoted antigen-specific IL-10 and IL-17-producing $CD8^+$ T cells, and enhanced the relative numbers of $CD4^+CD25^+Foxp3^+$ T regulatory cells.

Materials and Methods

Mice

6–8 weeks old C57BL/6 (Ly5.2) mice were purchased from Charles River Laboratories, Germany. C57BL/6 (Ly5.1) and OT-I mice were a generous gift of Marek Kovar, Institute of Microbiology of ASCR, v.v.i. OT-II mice were a generous gift of Thomas Jacobs, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany and Pavel Otahal, Institute of Molecular Genetics of ASCR. All of the experimental procedures were approved by the Animal Welfare Committee at the Institute of Microbiology of ASCR in accordance with institutional and state guidelines on animal welfare and every effort was made to minimize suffering.

Generation of mouse and human dendritic cells

Bone marrow-derived DCs (BMDCs) were generated according to Lutz et al. (1999) [27]. Briefly, bone marrow cells were flushed from femurs and tibias of mice, and cultured at 2×10^6/ml in 100-mm dishes in 10 ml of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Life Technologies), 0.1 mg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin (Sigma-Aldrich), 50 μM 2-mercaptoethanol, 1% non-essential amino acids (Biochrom), 1 mM sodium pyruvate, 2 mM glutamine and 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech). Fresh medium was added on day 3 or changed on day 6. Loosely attached cells were used for experiments at days 6–8. 70–80% of cultured cells at day 6–8 expressed CD11c and 90% CD11b. Before performing experiments, the BMDC's phenotype was checked for expression levels of CD11c+, CD11b+, I-A/I-E+, Gr-1, F4/80 and B220 low/negative (Fig. S1A).

Immature monocyte-derived DCs (MDDCs) were generated as previously described [28]. Briefly, human PBMC were isolated from buffy coats of healthy donors (provided by the Department of Blood Transfusion at Thomayer's Hospital, Prague, Czech Republic or Institute of Hematology and Blood Transfusion, Prague, Czech Republic) by Ficoll-Paque plus gradient centrifugation (GE Healthcare). PBMC at the concentration of 3×10^6 cells/ml were incubated in 75 cm^2 plastic culture flasks (Nunc). After 2 h, the non-adherent fraction of cells was washed away and isolated adherent monocytes were cultured in the presence of human GM-CSF (500 U/ml; Gentaur) and recombinant human IL-4 (20 ng/ml; PeproTech) in RPMI 1640 (BioWhittaker, Lonza), supplemented with L-glutamine (2 mM, Sigma), penicillin/streptomycin (100 U penicillin/ml, 100 μg streptomycin/ml), and 10% FCS (BioWhittaker, Lonza) at 37°C. Immature DCs were harvested on day 5 of culture. The phenotype of MDDCs had been verified before starting experiments (CD11c+, HLA-DR+, CD1a+, CD14 low/negative) (Fig. S1B). To avoid uncontrollable chelation of calcium ions by the phosphate ions contained in RPMI 1640 medium, DC stimulations were performed in DMEM medium as calcium is required for CyaA activity.

Purification of CyaA and determination of intracellular cAMP and cell viability

Wild type CyaA and CyaA-AC^- (CyaA mutant devoid of adenylate cyclase activity) were purified as described from *E. coli* [29]. The endotoxin content in samples was determined by the Limulus amebocyte lysate assay (QCL-1000; Cambrex) and was below 200 EU/mg of purified protein.

For determination of intracellular cAMP, BMDCs or MDDCs (2×10^5/sample) were seeded in 96-well plate in RPMI media containing 10% FCS and allowed to attach for 2 h at 37°C. Subsequently, RPMI media with 10% FCS was replaced by 150 μl DMEM without FCS and the CyaA at 10 ng/ml was added for 30 min. The reaction was stopped by addition of 0.2% Tween 20 in 50 mM HCl, and the samples were boiled for 15 min at 100°C to denature cellular proteins (cAMP is resistant to acid and heat). The samples were neutralized by addition of 150 mM unbuffered imidazole, and the concentration of cAMP was determined by a competition ELISA performed as previously described [30].

To determine cell viability, BMDCs (3×10^5/sample) or MDDCs (1×10^6/sample) were left untreated or incubated for 18 h or 24 h, respectively, with 10 ng/ml CyaA or CyaA-AC^- and LPS (1 μg/ml for MDDCs or 100 ng/ml for BMDCs, *E. coli* 0111:B4, Sigma-Aldrich) and subsequently stained with Annexin-

V-FITC (BD Pharmingen) and 0.5 μg/ml Hoechst 33258 (Invitrogen). The necrotic and/or apoptotic cells were detected by flow cytometry using FACS Aria (MDDCs) or LSR II instruments (BD Biosciences) (BMDCs) and analyzed by flow cytometry software (FlowJo Version 8.8.7, Tree Star, Inc.). In some experiments higher concentrations of 100 ng/ml or 300 ng/ml of CyaA and CyaA-AC$^-$ were used.

Determination of cell adhesion and spreading

To determine the adhesion and spreading of MDDCs impedance measurements using xCelligence system in E-plates (Roche) were performed. The increase in cell spreading and adhesiveness leads to increase in impedance, as cells attached on the electrode sensor surfaces act as insulators [31]. E-plates were coated with fibronectin in PBS for 1 h at room temperature, washed with PBS and background was determined in 90 μl of DMEM medium supplemented with 10% FCS using Real-Time Cell Analyzer (RTCA) station. Subsequently, MDDC suspension in DMEM medium (1×10^5/well), and LPS (1 μg/ml) alone or in combination with 10 ng/ml CyaA or CyaA-AC$^-$ was added. Cells in E-plates were placed in the RTCA station for 24 h cultivation at 37°C in a 5% CO_2 atmosphere. Impedance was measured every 5 min for an initial 5 h period of cultivation, and every 15 min for the remaining period of 19 h. Impedance was represented by the cell index (CI) values (R_i-R_0) [Ohm]/15 [Ohm]; R_0: background resistance, R_i: individual time point resistance).

DC migration

DCs were left untreated, or treated with LPS (1 μg/ml for MDDCs or 100 ng/ml for BMDCs) alone or in combination with 10 ng/ml CyaA or CyaA-AC$^-$ at cell density 1×10^6/ml in DMEM medium supplemented with 10% FCS for 24 h. Subsequently, cells were washed and their migration was measured in 96-well Transwell cell culture chambers with 5 μm pore size polycarbonate filters (Corning Costar). The lower chambers of the Transwell plates were filled with 235 μl of RPMI 1640 medium with or without CCL19 or CCL21 (200 ng/ml; Peprotech), and in total, 1×10^5 DCs diluted in 75 μl of RPMI medium were deposited in the upper chamber. After 14 h (MDDCs) or 4 h (BMDCs) of incubation at 37°C in a 5% CO_2 atmosphere, the Transwell inserts were removed. DCs in the lower chamber were transferred into a new 96-well plate, stained with Hoechst 33258 and live cells were counted by flow cytometry. CCR7 expression was determined by using CCR7-PE antibody (eBiosciences).

Measurement of antigen uptake, processing and degradation

To measure antigen (Ag) uptake, DCs (3×10^5/sample) were left untreated, or pretreated with LPS (100 ng/ml) and 10 ng/ml of CyaA or CyaA-AC$^-$ for 30 min. Subsequently, OVA-Alexa647 or transferrin-Alexa647 (both 5 μg/ml, Invitrogen) or Lucifer yellow (500 μg/ml, Invitrogen) were added for 30 min at 37°C. The Ag uptake was assessed by flow cytometry.

For MHC class I-restricted processing DCs (2×10^6/sample) were left untreated, or incubated with 10 ng/ml of CyaA or CyaA-AC$^-$ ng/ml or lactacystin (10 μM, Sigma-Aldrich) and LPS (100 ng/ml) for 30 min. Protein concentration in lysate was determined by MicroBCA™ Protein Assay kit (Pierce). 50 μg of proteins in 20 mM Tris-HCl, pH 7.4 was mixed with 100 μM Z-Leu-Leu-Glu-AMC, Suc-Leu-Leu-Val-Tyr-AMC or Boc-Leu-Arg-Arg-AMC fluorogenic substrates (BIOMOL) and incubated for 90 min at 37°C. The fluorescence of liberated 7-amino-4-methylcoumarin was measured using a microplate reader (380_{ex}/460_{em}, Safire^2, Schoeller Instruments).

For MHC class II-restricted Ag processing DCs (1×10^6/sample) were left untreated, or incubated with 10 ng/ml of CyaA or CyaA-AC$^-$ or chloroquine (100 μM, Sigma-Aldrich) and LPS (100 ng/ml) for 30 min. DCs were subsequently loaded with a mixture of OVA-Alexa647 and OVA labeled with BODIPY FL dye (OVA-DQ, both 5 μg/ml, Invitrogen) for 30 min at 37°C and analyzed by flow cytometry [32].

T cell expansion *in vitro* and *in vivo*

For T cell proliferation *in vitro* naïve OVA-specific CD8$^+$ or CD4$^+$ T cells were isolated from lymph nodes and spleen of OT-I or OT-II transgenic mice, respectively, by a magnetic cell separation using CD8$^+$ and CD4$^+$ T cell Isolation kits (Miltenyi) and labeled with 3 μM CFSE (Invitrogen) for 10 min, at 37°C. 2×10^5 T cells were added to washed 5×10^4 DCs which had been left untreated, or pretreated with LPS (100 ng/ml) and 10 ng/ml of CyaA or CyaA-AC$^-$ and OVA protein at 2.5 μg/ml (OT-II) or at 5 μg/ml (OT-I) for 4 h at 37°C. In some experiments 5 μg/ml $OVA_{323–339}$ peptide (ISQAVHAAHAEINEAGR, GeneScript) or 1 ng/ml $OVA_{257–264}$ peptide (SIINFEKL) were added instead of the OVA protein for 4 h at 37°C. After 72 h of incubation T cells were stained by CD8-PE (53–6.7, eBioscience) or CD4-PE (RM4-4, eBioscience) antibodies and their proliferation was determined as a CFSE dilution by flow cytometry.

For T cell expansion *in vivo*, purified CFSE-labeled T cells (Ly5.2) were injected intravenously (i.v.) into C57BL/6 recipients (Ly5.1) at 1.5×10^6 cells per mouse. 24 h later mice were injected i.v. with 1.5×10^6 DCs that had been pretreated with toxin, LPS and OVA peptides as described above. Control mice were injected intraperitoneally (i.p.) with PBS or OVA peptide+poly I:C (75 μg). Three days later, spleen cells were harvested, fixed by 4% paraformaldehyde and stained with CD8-A700 or CD4-PerCP and Ly5.2-APC antibody (BD Pharmingen). The expansion of adoptively transferred T cells was determined by flow cytometry.

MDDCs, derived from monocytes of HLA-A2 positive healthy donors, were left untreated, or stimulated with LPS (1 μg/ml) alone or in combination with 10 ng/ml of CyaA or CyaA-AC$^-$ at cell density 10^6/ml in DMEM medium supplemented with 10% FCS for 24 h. Subsequently, DCs were pulsed with the HLA-A2 restricted influenza matrix peptide (aa 58–66, GILGFVFTL) for 2 h, washed and added to autologous lymfocytes at T cell: DC ratio of 10 : 1 for 7 days. IL-2 (50 U/ml) was added on day 3. On day 7, lymphocytes were re-stimulated with fresh peptide-loaded DCs for 1 h and analyzed for IFN-γ production by intracellular flow cytometry staining, as follows: Brefeldin A (e-Bioscience, San Diego, CA, USA) was added to block the release of IFN-γ After 3 h cells were stained with CD3-Alexa700 and CD8-PE-Dy590 antibodies (Exbio) at 4°C for 20 minutes. Subsequently, cells were fixed and permeabilized using Fixation and Permeabilization Buffers (e-Bioscience), respectively, and stained with IFN-γ-FITC antibody (e-Bioscience). Samples were analyzed using flow cytometry.

T cell responses and expansion of CD4$^+$CD25$^+$Foxp3$^+$ T cells

BMDCs (5×10^4/sample) were left untreated, or treated with 10 ng/ml of CyaA or CyaA-AC$^-$ and/or LPS (100 ng/ml). Concomitantly OVA protein (2.5 μg/ml) was present for 4 h prior assays of MHC class II presentation or at 5 μg/ml prior assay of MHC class I presentation and incubated with T cells. Production of IL-17, IFN-γ and IL-10 by CD4$^+$ and CD8$^+$ T cells in supernatant was determined by ELISA after 72 h. Remaining

$CD4^+$ T cells were collected and stained with CD4-PerCP (BD Biosciences), CD25-APC and Foxp3-PE (eBioscience) using Fixation and Permeabilization buffers (eBioscience). The expression of Foxp3 in $CD4^+CD25^+$ T cells was determined by flow cytometry.

MDDCs were left untreated or incubated with LPS (1 μg/ml) alone or in combination with CyaA and CyaA-AC^- (10 ng/ml) for 24 h and used as stimulators of naïve allogeneic T cells, isolated by a negative selection using EasySep Human Naïve $CD4^+$ T Cell Enrichment Kit (StemCell Technologies Inc.). T cells were used at T cell : DC ratio of 10 : 1 and IL-2 (50 U/ml) was added on day 3. After 7 days of co-culture, the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ T regulatory cells was determined by flow cytometry. Cells were stained with CD8-PE-Dy590, CD25-PE (Exbio) and CD4-PE-Cy7 (eBioscience) antibodies at 4°C for 30 minutes, followed by cell fixation and permeabilization using Fixation and Permeabilization Buffers (e-Bioscience), respectively and staining with Foxp3-Alexa488 antibody (e-Bioscience). The gating strategy of T regulatory cells is shown in Fig. S4.

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using software GraphPad (PRISM 6.0). The significance of the differences between groups was determined by using two-tailed Mann-Whitney U test. Differences were considered statistically significant if $p<0.05$ (*).

Results

Exposure to low concentration of CyaA does not induce cell death in TLR-activated DCs

CyaA used at high concentrations was shown to induce cell death [12,13]. Therefore, prior to analyzing the effect of CyaA on immunostimulatory activities of TLR-stimulated DCs, we first examined the impact of exposure to low CyaA concentration (10 ng/ml) on viability of murine BMDCs and human MDDCs. As shown in Fig. 1A, upon 30 min of incubation with 10 ng/ml of CyaA, the BMDCs and MDDCs accumulated similarly high levels of intracellular cAMP. Fig. 1B then shows that the treatment of MDDCs with 10 ng/ml of CyaA in the absence of LPS led to a significant cell death in 24 hours. However, co-treatment with LPS rescued MDDCs from CyaA-induced cell death. On the contrary, mouse BMDCs were more resistant to CyaA-induced cell death in the absence of LPS, as CyaA at 10 ng/ml did not reduce BMDC's viability (Fig. 1C). However, LPS treatment decreased the survival of BMDCs by 20% and the co-treatment of BMDCs with LPS and CyaA at 10 ng/ml rescued DCs from LPS-induced cell death. These effects were mediated by CyaA-induced cAMP signaling, since the enzymatically inactive CyaA-AC^- did not affect cell viability of untreated, or LPS-treated MDDCs and BMDCs, respectively. Fig. 1D further shows that CyaA at the higher concentrations of 100 ng/ml and 300 ng/ml induced cell death in BMDCs independently of LPS-signaling. The cytotoxic effects of LPS or CyaA varied depending on the origin of DCs. Interestingly, the co-incubation with both CyaA (10 ng/ml) and LPS lead to pro-survival signaling in both types of DCs, MDDCs and BMDCs.

CyaA accelerates TLR-mediated dissolution of cell adhesive contacts and migration of DCs

We have previously observed that as low doses of CyaA as 10 ng/ml induce massive actin cytoskeleton rearrangements and ruffling of mouse macrophages [14]. Therefore we analyzed the effects of exposure to low doses of CyaA (10 ng/ml) on the immune functions of DCs. Modulation of human or mouse DC maturation and IL-12p70 and IL-10 cytokine production by CyaA has been extensively analyzed *in vitro* using either the *E. coli* LPS, or in the context of DC infection by *B. pertussis* strains [15–19]. CyaA at higher concentrations was shown to inhibit LPS-stimulated CD40 expression and IL-12p70 production and enhance IL-10 secretion from both mouse and human DCs [15–19]. As shown in Fig. S2, treatment with CyaA (10 ng/ml) reproduced the previously reported impact of CyaA action on DC phenotype and cytokine production in the presence of LPS [15–19]. We extended, therefore, our analysis to CyaA effects on activation, adhesive and migratory properties of DCs after TLR-stimulation. We first assessed DC adhesion and spreading by impedance measurements using the xCelligence system [31]. MDDCs were left untreated, or treated with LPS alone, or in combination with CyaA or CyaA-AC^- at 10 ng/ml for 24 h. As shown in Fig. 2A, the impedance measurements revealed that LPS stimulation of MDDCs resulted in rapid increase of cell adhesion and spreading, as detected by a rise in the cell index (CI). This was followed by drop of the CI value below that of control non-treated cells (medium) within 24 h, due to progressing maturation and cell rounding up. Importantly, addition of CyaA to LPS-activated MDDCs resulted in a faster decrease of cell adhesion and spreading, as compared to LPS stimulated cells and cells treated with LPS and non-enzymatic CyaA-AC^- toxoid (Fig. 2A). Fig. 2B then depicts the CI values of the samples at 12 h of the impedance measurements. These results suggest that through elevation of cAMP the CyaA action accelerates TLR-induced decrease of cell adhesion that accompanies DC maturation.

Reorganization of the cytoskeleton and dissolution of cell adhesive contacts during DC maturation is important for the capacity of DCs to migrate into the lymph nodes. Therefore, we next examined whether the increased dissolution of cell adhesion mediated by CyaA also leads to an increased migration of TLR-stimulated DCs to CCL19 or CCL21 chemokines. BMDCs or MDDCs were exposed for 24 h to LPS alone or in combination with 10 ng/ml of CyaA or CyaA-AC^- and the cells were allowed to migrate across a transwell membrane into medium with chemokines for additional 14 h (MDDCs) or 4 h (BMDCs), respectively. As shown in Fig. 2C, CyaA increased chemotactic responsiveness of LPS-stimulated DCs to CCL19 or CCL21 chemokines compared to cells treated with LPS and CyaA-AC^- toxoid. However, after 24 h of treatment with CyaA and LPS, the DCs did not express significantly higher amounts of the CCL19/21 chemokine receptor CCR7 than DCs treated with LPS alone (Fig. 2D). As shown in Fig. 2D, a significantly increased expression of CCR7 on DCs treated with LPS and CyaA toxin was observed only after 48 h of incubation.

CyaA decreases the capacity of TLR-stimulated DCs to present soluble antigens to $CD4^+$ T cells

CyaA differentially modulates LPS-stimulated DC maturation and cytokine production [16,18,19], which may affect their capacity to stimulate T cells. Therefore, we further investigated the capacity of CyaA-treated DCs to stimulate proliferation of $CD4^+$ T cells. BMDCs were left untreated, or treated with 10 ng/ml of CyaA or CyaA-AC^- in the presence of ovalbumin (OVA) and LPS for 4 h. Toxin was removed by cell washing and naïve OVA-specific OT-II $CD4^+$ T cells were then added. As shown in Fig. 3A and 3B, pretreatment of DCs with CyaA decreased their capacity to stimulate T cell proliferation, as higher percentage of undivided OT-II $CD4^+$ T cells (54%) was detected by a CFSE dilution assay at 72 hours after $CD4^+$ T cell incubation with CyaA-treated and OVA-loaded DCs, than after incubation with

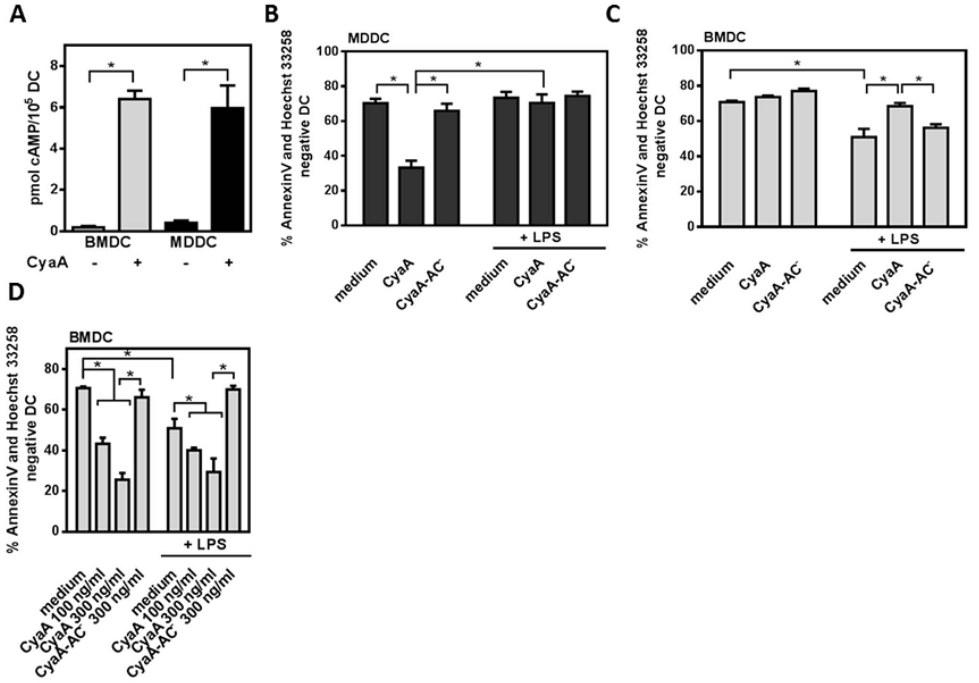

Figure 1. Exposure to low concentrations of CyaA does induce cell death in TLR-activated DCs. (A) BMDCs and MDDCs were left untreated or incubated with CyaA at 10 ng/ml for 30 min. The intracellular level of cAMP was determined by ELISA. (B, C) DCs were left untreated (medium), or incubated with LPS (1 μg/ml MDDCs or 100 ng/ml BMDCs), CyaA or CyaA-AC⁻ at 10 ng/ml alone or in their combination for 18 h (BMDCs) or 24 h (MDDCs) (D) BMDCs were incubated with LPS and CyaA or CyaA-AC⁻ at 100 ng/ml or 300 ng/ml alone or in their combination for 18 h and stained with Annexin V-FITC and Hoechst 33258. Values represent the means ± SEM of n = 3–5 or 5–6 donors, respectively (* $p<0.05$).
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DCs treated with LPS only (37.2%), or with the CyaA-AC⁻ plus LPS-treated DCs (39.9%), respectively.

To investigate whether absence of co-stimulation or the CyaA-induced production of inhibitory soluble factors like IL-10 (Fig. S2) accounted for the reduced capacity of CyaA-pretreated DCs to stimulate $CD4^+$ T cells, we used CyaA-pretreated DCs loaded with specific OVA peptide, which in contrast to a protein antigen does not require processing for T cell presentation. As further shown in Fig. 3A and 3B, the CyaA-pretreated DCs loaded with the OVA peptide stimulated even higher $CD4^+$ T cell proliferation than control LPS- or CyaA-AC⁻-treated DCs. Similar data were obtained in an assay that assessed the expansion of adoptively transferred OT-II $CD4^+$ T cells *in vivo* (Fig. 3C), where LPS-stimulated and CyaA-pretreated and OVA peptide-pulsed DCs induced higher proliferation of $CD4^+$ T cells than control cells treated with LPS. This indicates that cAMP signaling of CyaA did not affect the overall capacity of LPS-treated DCs to stimulate $CD4^+$ T cells when loaded with the OVA-derived peptide. However, the decrease in protein antigen presentation suggests that CyaA/cAMP may have affected antigen processing in LPS-stimulated DCs.

Using J774 macrophages we have previously shown that CyaA inhibited macropinocytosis in CD11b-expressing phagocytes [14] and this may have also impacted on presentation of OVA protein to T cells by DCs [33]. Therefore, we analyzed if CyaA action inhibits uptake of OVA. At the highest OVA concentration used in our study (5 μg/ml) the uptake of OVA was mediated solely by receptor-mediated endocytosis (Fig. S3). At higher concentrations, however, OVA could be internalized by both, macropinocytosis and receptor-mediated endocytosis [33]. We examined separately the effect of CyaA activity on macropinocytosis in DCs and on receptor-mediated endocytosis using Lucifer Yellow or Transferrin-Alexa647 uptake assays, respectively. BMDCs were left untreated, or exposed to 10 ng/ml of CyaA or CyaA-AC⁻ for 30 min in the presence of LPS, and then incubated with Ags for additional 30 min before being analyzed by flow cytometry. As shown in Fig. 3D, while CyaA action inhibited macropinocytosis of Lucifer yellow by ~60%, it did not affect receptor-mediated endocytosis of transferrin in DCs compared to CyaA-AC⁻ and LPS-treated cells.

Since elevated cAMP levels were previously reported to reduce Ag degradation capacity of macrophages [34], we next examined if CyaA could inhibit Ag degradation in DCs. To analyze MHC class II-restricted processing, LPS and CyaA-treated DCs were incubated with a mixture of OVA-Alexa647, used as a marker for Ag uptake, and OVA labeled with BODIPY®FL dye (OVA-DQ),

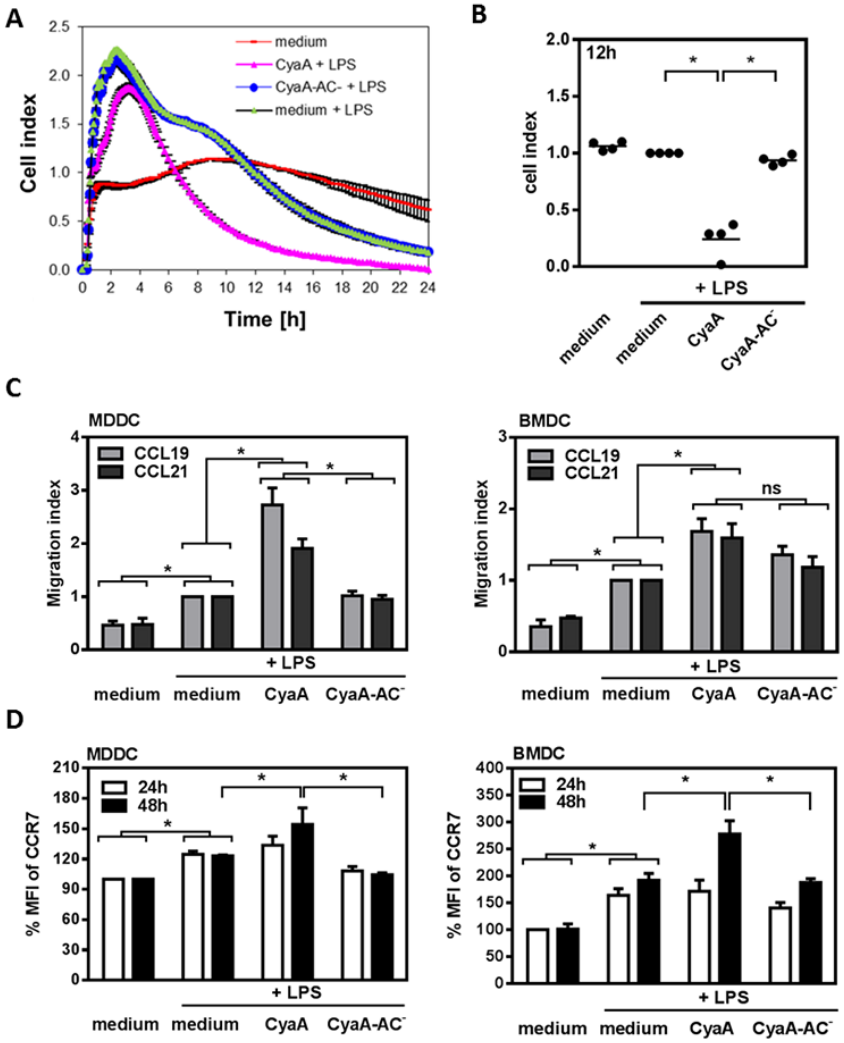

Figure 2. CyaA accelerates cell detachment and migration of TLR-activated DCs. (A) Impedance measurements using the real-time cell electronic sensing system xCelligence were used to determine MDDC adhesion and spreading. MDDCs were seeded on fibronectin-coated sensors and were left untreated (medium), or treated with LPS (1 μg/ml) alone, or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml for 24 h. The representative experiment is shown (A) as well as quantitative analysis of 4 donors at time point of 12 h (B) where cell index (CI) of LPS-treated DCs at 12 h was normalized to 1.0. (C) Migration of DCs treated with toxins and LPS (for 24 h) towards CCL19 or CCL21 (both 200 ng/ml) in transwell plates was determined by flow cytometry after additional 14 h (MDDCs) or 4 h (BMDCs) of incubation at 37°C. Values represent the means ± SEM of n = 4 or 5 donors, respectively (* $p<0.05$) where the number of transmigrated LPS-treated DCs (medium) was set to 1. (D) CCR7 expression on DCs was determined by flow cytometry after 24 h and 48 h. Values represent the means ± SEM of n = 3–5 or 5 donors, respectively (* $p<0.05$).
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which served as a marker for Ag uptake and processing. Calculation of OVA-DQ degradation, was then determined as a ratio of % MFI OVA-DQ/% MFI OVA-Alexa647 by flow cytometry. As shown in Fig. 3E, however, only a slight and insignificant decrease in OVA-DQ degradation in DCs exposed to CyaA (0.78%±0.2) was observed, as compared to treatment with

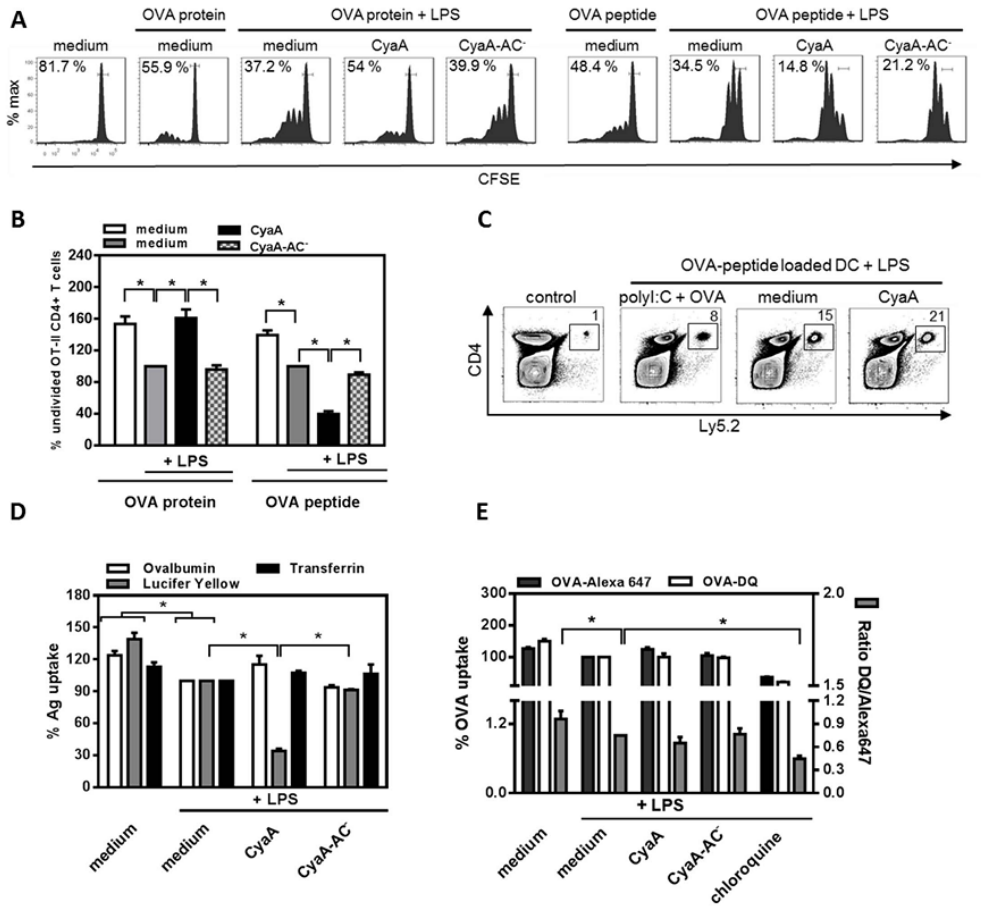

Figure 3. CyaA decreases the capacity of TLR-stimulated DCs to present soluble antigen to CD4$^+$ T cells. BMDCs were left untreated, incubated with LPS (100 ng/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml in the presence of OVA protein at 2.5 μg/ml or $OVA_{323–339}$ peptide (5 μg/ml) for 4 h prior to washing and co-cultivation with naïve CFSE-labeled OT-II CD4$^+$ T cells. T cell proliferation was determined by flow cytometry after 72 h as a dilution of CFSE. (A) Histograms are representative of n = 4. (B) Quantitative analysis of A where the percentage of undivided LPS-treated cells (medium) was set to 100% (* $p<0.05$). (C) Expansion of adoptively transferred CFSE-labeled CD4$^+$ T cells *in vivo* was determined after 72 h by flow cytometry as a fold of expansion of 2×10^6 counted spleen cells where 1 represents the non-stimulated adoptively transferred CD4$^+$ T cells (control). Dot plots are representative of n = 3. (D, E) CyaA inhibits macropinocytosis but not receptor-mediated endocytosis and antigen (Ag) degradation in LPS-treated DCs. DCs were left untreated, incubated with LPS alone or in combinantion with 10 ng/ml of toxins or chloroquine (100 μM) for 30 min. (D) Lucifer Yellow (500 μg/ml), transferrin-Alexa647 or OVA-Alexa647 (both 5 μg/ml) were subsequently added for 30 min. The Ag uptake in living CD11c$^+$ cells was determined by flow cytometry. (E) A mixture of OVA-Alexa647 (5 μg/ml, marker for Ag uptake) and OVA-DQ (5 μg/ml, marker for Ag uptake and degradation) were added for 30 min. The processed OVA-DQ was determined from gated CD11c$^+$OVA-DQ$^+$OVA-Alexa647$^+$ DCs and calculated as a ratio of MFI OVA-DQ/OVA-Alexa647. Values represent means ± SEM of n = 5 where Ags taken up by LPS-treated DC (medium) was set to 100% of MFI (ratio 1) (* $p<0.05$).
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CyaA-AC$^-$ toxoid (1.01%±0.2),), or to non-treated cells (1.0%±0.0). In contrast, endosomal processing was inhibited upon treatment with chloroquine (0.59%±0.05). These findings suggest that CyaA shuts down macropinocytosis but not the receptor-mediated endocytosis of OVA. As CyaA displayed no inhibitory effect on OVA uptake and degradation in LPS-stimulated DCs, the inhibition of OVA protein presentation to CD4$^+$ T cells by CyaA-treated DCs is likely to involve other steps of antigen processing and presentation pathway within the DCs.

CyaA commits DCs to expand CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cell population *in vitro*

LPS-stimulated DCs treated with CyaA were shown to polarize IL-17-producing CD4$^+$ T cells while reducing INF-γ production in T cells [18]. CyaA was also shown to induce IL-10 producing Tr1 cells [18]. However, it is not clear if LPS-stimulated DCs treated with CyaA expand a distinct subset of T regulatory cells, such as CD4$^+$CD25$^+$Foxp3$^+$ T cells [35]. Therefore, BMDCs were left untreated, or incubated with LPS alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml in the presence of OVA protein for 4 h before isolated naïve OVA-specific OT-II CD4$^+$ T cells were added for another 72 h. The gating strategy used to detect both, human and mouse CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells, is shown in Fig. S4. As documented in Fig. 4A by the results of flow cytometry analysis, CyaA significantly increased the capacity of LPS-stimulated BMDCs to expand OVA-specific CD4$^+$CD25$^+$Foxp3$^+$ T cells as compared to CyaA-AC$^-$-treated DCs.

Similarly, MDDCs were left untreated, or incubated with LPS alone, or in combination with CyaA or CyaA-AC$^-$ (10 ng/ml) for 24 h before use for stimulation of naïve allogeneic T cells. Frequency of CD4$^+$CD25$^+$Foxp3$^+$ T cells was measured by flow cytometry after 7 days. As shown in Fig. 4B, LPS and CyaA-treated MDDCs expanded the relative number of CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells from co-cultivated CD4$^+$ T cells. Collectively, these data show that CyaA-induced cAMP signaling skews the TLR-stimulated DCs towards the expansion of CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells *in vitro*.

CyaA reduces the capacity of TLR-stimulated DCs to induce CD8$^+$ T cell proliferation

To assess if the CyaA/cAMP-induced modulation of LPS-stimulated DCs impacts on activation of CD8$^+$ T cell responses, BMDCs were left untreated, or pretreated with 10 ng/ml CyaA, or with CyaA-AC$^-$, in the presence of OVA protein or peptide and LPS for 4 h. Residua ltoxin was removed by cell washing and naïve OT-I CD8$^+$ T cells were added. As shown in Fig. 5A and 5B, treatment with CyaA interfered with the capacity of DCs to stimulate proliferation of CD8$^+$ T cells. Higher percentage of undivided OT-I CD8$^+$ T cells was detected by a CFSE dilution assay after incubation of CyaA-treated DCs for 72 h with OVA protein (66.8%), or with OVA peptide (72.6%), as compared to control LPS-treated DCs (26.5% or 35.9%, respectively) and the CyaA-AC$^-$-treated DCs (30.6% or 40.3%),%), respectively. CyaA also decreased the capacity of LPS-stimulated DCs to induce proliferation of OT-I CD8$^+$ T cells in response to OVA peptide after adoptive transfer *in vivo*, as compared to control LPS-treated and OVA peptide pulsed DCs (Fig. 5C).

Similarly, MDDCs, derived from monocytes of HLA-A2 positive healthy donors, were left untreated, or incubated with LPS alone or in combination with 10 ng/ml CyaA or CyaA-AC$^-$ for 24 h. Subsequently, MDDCs were pulsed with the HLA-A2

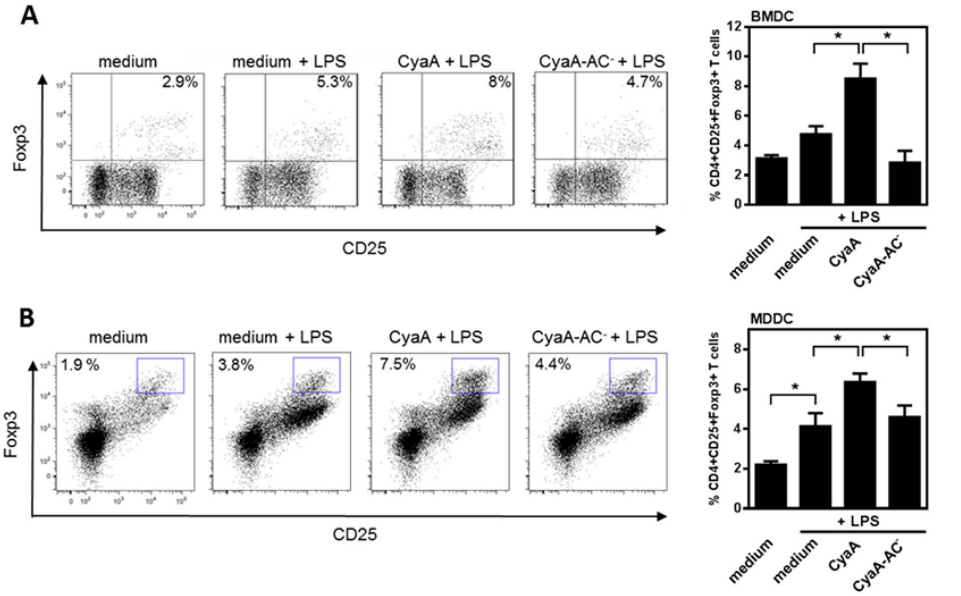

Figure 4. CyaA commits TLR-stimulated DCs to expand CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells *in vitro*. (A) BMDCs were left untreated, incubated with LPS (100 ng/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml in the presence of OVA at 2.5 μg/ml for 4 h prior to washing and co-cultivation with naïve CFSE-labeled OT-II CD4$^+$ T cells. After 72 h the number of CD4$^+$CD25$^+$Foxp3$^+$ T cells was determined by flow cytometry. Dot plots show one representative experiment and quantitative analysis represent means ± SEM of n = 4 (* $p<0.05$). (B) MDDCs were incubated with LPS (1 μg/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml. After 24 h cells were used as stimulators of naïve allogeneic T cells at DC : T cell ratio of 1 : 10. The expansion of human CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells was determined after 7 days. The representative experiment is shown and quantitative analysis represent means ± SEM of n = 5 (* $p<0.05$).
doi:10.1371/journal.pone.0104064.g004

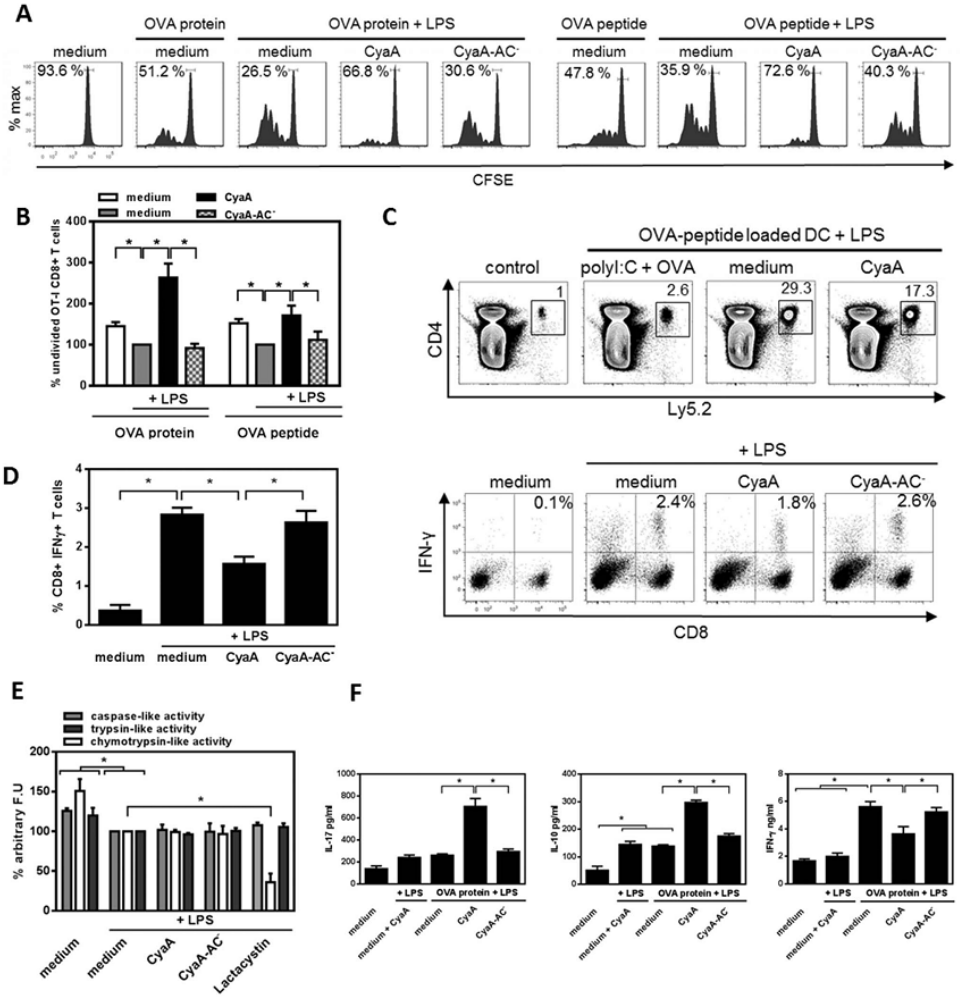

Figure 5. CyaA reduces the capacity of TLR-stimulated DCs to induce CD8$^+$ T cell proliferation. BMDCs were left untreated, incubated with LPS (100 ng/ml) alone or in combination with 10 ng/ml of CyaA or CyaA-AC$^-$ in the presence of OVA protein at 5 μg/ml or $OVA_{257–264}$ peptide (1 ng/ml) for 4 h prior to co-cultivation with naïve CFSE-labeled OT-I CD8$^+$ T cells. T cell proliferation was determined by flow cytometry after 72 h as a dilution of CFSE. (A) Histograms are representative of n = 4. (B) Quantitative analysis of A where the percentage of undivided LPS-treated cells (medium) was set to 100% (* $p<0.05$). (C) Expansion of adoptively transferred CFSE-labeled CD8$^+$ T cells *in vivo* after 72 h was determined by flow cytometry as a fold of expansion of 2×10^6 spleen cells counted where 1 represents the non-stimulated adoptively transferred CD8$^+$ T cells (control). Dot plots are representative of n = 3 (D) MDDCs were incubated with LPS (1 μg/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml for 24 h and then loaded with influenza matrix peptide. The induction of specific IFN-γ producting CD8$^+$ T cells was determined after 7 days by flow cytometry. Dot plots show one representative experiment and quantitative analysis represent means ± SEM of n = 6 (* $p<0.05$). (E) BMDCs were left untreated, incubated with LPS (100 ng/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml or inhibitor of proteasome lactacystin (10 μM) for 30 min. After cell lysis, 50 μg of cellular proteins was mixed with fluorogenic proteasomal peptide substrates (100 μM) and incubated 90 min at 37°C. Values represent means ± SEM of n = 4 where the amount of processed substrates by LPS-treated DCs (medium) was set to 100% (* $p<0.05$). (F) The production of IL-17, IL-10 and IFN-γ in BMDC-CD8$^+$ T cell culture supernatant after 72 h was determined by ELISA. Mean values ± SEM are representative of n = 3 (* $p<0.05$).

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restricted influenza matrix peptide (aa 58–66, GILGFVFTL) for 2 h, washed and incubated with autologous T lymphocytes for 7 days. IFN-γ-producing T cells were detected by flow cytometry after restimulation with such treated DCs. As shown in Fig. 5D, pretreatment with CyaA decreased the capacity of LPS-activated MDDCs to stimulate influenza specific $CD8^+$ T cells compared to control LPS-treated or CyaA-AC^- and LPS-treated MDDCs, respectively.

To assess if CyaA impairs the MHC class I-restricted processing in DCs and this may account for the observed decrease in $CD8^+$ T cell proliferation, lysates of BMDCs pretreated with CyaA or with CyaA-AC^- were incubated with fluorescent peptide substrates for 90 min and the proteolytic activities of the proteasome were determined as the intensity of resulting fluorescence. As shown in Fig. 5E, CyaA did not inhibit chymotrypsin-like, trypsin-like or caspase-like proteolytic activity of the proteasome, while this was inhibited by lactacystin, a known inhibitor of proteasomal proteolytic functions. These findings indicate that CyaA diminished the capacity of antigen-loaded DCs to stimulate $CD8^+$ T cell proliferation *in vitro* as well as *in vivo*, which appeared to be independent of proteosomal Ag processing for MHC class I presentation.

In addition, the analysis of cytokine production in BMDC-$CD8^+$ T cell culture supernatants by ELISA revealed that LPS and CyaA-pretreatment of DCs polarized the OVA-specific IL-17 and IL-10 production by $CD8^+$ T cells, but suppressed production of IFN-γ (Fig. 5F). This goes well with the cytokine profiles induced by LPS and CyaA-pretreated DCs in $CD4^+$ T cells (Fig. S5). These findings show that cAMP signaling of CyaA in DCs directs also $CD8^+$ T cells to produce IL-17 and IL-10 and limits the antigen-specific production of IFN-γ in response to stimulation by LPS-activated DCs.

Discussion

Through the elevation of cytosolic cAMP concentrations, the CyaA toxin exerts a remarkably complex set of effects on immune cell functions. This ranges from inhibition of phagocytosis [10], oxidative burst [11], or macropinocytic uptake [14], up to a direct cytotoxicity and apoptotic [12] or necrotic cell death [13]. Here we show that human monocyte-derived DCs (MDDCs), but not mouse bone marrow-derived DCs (BMDC), are highly sensitive to CyaA-mediated cytotoxicity already at as low concentration of CyaA as 10 ng/ml and that this cytotoxicity of CyaA could be counteracted by co-incubation with LPS. On the other hand, BMDCs were slightly more sensitive to LPS treatment and the addition of CyaA increased their survival rate *in vitro*. Interestingly, in the absence of LPS or other TLR ligands, CyaA was shown to induce phenotypic maturation of DCs and to enhance their capacity to stimulate T cells in mixed lymphocyte reaction [15]. Similarly, we have observed here that CyaA at 10 ng/ml also induced phenotypic maturation of BMDCs and MDDCs in the absence of TLR-stimulation (Fig. S6). However, this effect of LPS-free CyaA alone appears to be rather artificial, since in the context of *B. pertussis* infection a number of TLR ligands is shed by the infecting bacteria and the concerted action of other virulence factors would integrate into the final outcome as well. The main aim of this study was to analyze how low concentrations of CyaA shape the immune function of LPS-stimulated MDDCs and BMDCs. We confirmed that cAMP signaling induced by 10 ng/ml of *B. pertussis* CyaA toxin enhanced secretion of IL-10 and decreased production of IL-12p70 and CD40 expression by TLR-activated human and mouse DCs [16,18,19]. Moreover, as further shown here, it also enhanced TLR-induced cell detachment and chemotactic migration of DCs towards the chemokines CCL19/21. Furthermore, such subversively matured DCs exhibited a reduced capacity to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells and were able to expand $CD4^+CD25^+Foxp3^+$ T regulatory cells. As the key function of DCs is to promote differentiation of naïve T cells [36], the targeting of DCs by CyaA might represent a strategy of *B. pertussis* towards dampening of the adaptive immune response in the course of infection. Indeed, some subsets of DCs appear to have an important role in protective immunity to respiratory infection with *B. pertussis* [37].

We further report here that CyaA-induced cAMP signaling enhances LPS-mediated dissolution of cell adhesive contacts and promotes chemotactic migration of DCs towards CCL19/21 chemokines. However, we did not observe a correlation between the amount of CCR7 on the DC cell surface after 24 h and the enhanced chemotactic migration of CyaA and LPS-treated DCs, suggesting that the LPS-induced CCR7 expression was sufficient for enhanced migration along the cytokine gradient. Indeed, as documented by the xCelligence measurements, DCs treated with CyaA and LPS exhibited a faster dissolution of adhesive contacts, possibly facilitating migration. It has been shown that actin and myosin inhibitors inhibit the speed of migration but not the directed motion [38] and the integrin-ligand binding properties of cells similarly affects the migration speed [39]. It is therefore possible, that the enhanced motility induced by CyaA in LPS-treated DCs accounts for the higher number of transmigrated DCs in transwell experiments, despite the similar levels of CCR7 expression. It remains to be established, however, to which extent the capacity of CyaA to increase migration might play a role during *B. pertussis* infection *in vivo*, because wild-type *B. pertussis* was shown to inhibit migration of MDDCs towards CCL21 *in vitro* [40]. This was dependent on the presence of pertussis toxin, where the *B. pertussis* strains lacking active pertussis toxins promoted migration of MDDCs [40].

We further demonstrate here that CyaA-treated LPS-stimulated DCs displayed a decreased capacity to stimulate Ag-specific $CD4^+$ and $CD8^+$ T cells. This finding is consistent with the observations of Boschwitz and co-authors, who demonstrated that CyaA accounts for the suppressive activity of *B. pertussis*-infected monocytes on proliferation of Ag-specific $CD4^+$ T cells *in vitro* [41]. Moreover, inhibition of T cell responses has already been demonstrated in the context of *B. pertussis* infection, where T cells from lungs of *B. pertussis*-infected mice were found to be impaired in capacity to respond to *Bordetella* antigens [42]. Recently, it has also been shown that cAMP signaling skews human DC differentiation towards a tolerogenic phenotype and a defective T cell priming capacity [43]. We show here that the impairment of $CD4^+$ T cell stimulatory capacity of CyaA-treated DCs was neither due to a decrease in their viability, nor was it due to a decrease in expression of MHC class II and/or co-stimulatory molecules on the cell surface of toxin-treated DCs, or production of inhibitory soluble factors, like IL-10 or prostaglandins. It could, hence, be attributed to a defect in MHC II-restricted presentation of soluble OVA by CyaA-treated DCs, since upon loading with OVA peptide the CyaA-treated DCs enhanced proliferation of OVA-specific $CD4^+$ T cells both *in vitro* and *in vivo*. This all excludes a direct inhibitory impact of traces of CyaA in washed DC suspensions on T cell function [44]. Furthermore, we ruled out also the impact of a decreased macropinocytic uptake of OVA by DCs as the major cause of a reduced antigen presentation to T cells [33]. Most of OVA was used here at a low concentration and was thus taken up by the receptor-mediated endocytosis that was insensitive to CyaA/cAMP signaling. Moreover, no reduction in endosomal degradation of OVA in toxin-treated DCs was

observed either. Collectively, these results suggest that CyaA-mediated elevation of cellular cAMP concentrations interfered with some steps in the Ag presentation pathway that are downstream to Ag uptake and degradation. The most plausible explanation is that CyaA action caused perturbation of vesicular sorting and trafficking of epitope-loaded MHC II molecules from endosomes to the cell surface. This would go well with our previous observations that exposure to already as little as 10 ng/ml of CyaA does induce massive actin cytoskeleton rearrangements and membrane ruffling in CD11b-expressing myeloid cells [14]. In line with that, cAMP signaling induced by cholera toxin of *V. cholerae* and heat-labile enterotoxin of *E. coli* was also shown to impair presentation of protein or peptide antigens by macrophages or B cell lymphoma, albeit the mechanism has not been analyzed in detail [34,45,46].

It has previously been shown that naïve $CD8^+$ T cells require a "third signal", such as IL-12p70, for *in vitro* activation in response to antigenic peptides the [47]. The lack of IL-12p70 signaling may, indeed, explain the observed decrease in naïve $CD8^+$ T cell proliferation induced by CyaA-treated BMDCs. However, as IL-12p70 was shown to be dispensable for the activation of memory $CD8^+$ T cells [48], it is unlikely that the decrease of IFN-γ-production in human $CD8^+$T cells after stimulation with CyaA-treated MDDCs loaded with influenza matrix peptide would be due to a lack of IL-12p70 signaling. CyaA-induced reduction of MHC class I surface expression on BMDCs (Fig. S2) may, however, account for a decreased $CD8^+$ T cell stimulatory capacity of CyaA-treated DCs loaded with OVA peptide both *in vitro* and *in vivo*.

Exogenous peptides were shown to take a pinocytic pathway to reach endoplasmic reticulum (ER) [49]. It has further been previously shown that ER stress impairs MHC class I presentation of endogenous as well as of exogenously added peptides by a mouse lymphoma line cells [50]. Moreover, ER-stress induced miR-346 negatively regulates mRNA for the antigen peptide transporter 1 (TAP1), which might explain the reduced MHC class I presentation during ER-stress [51]. It is not known, however, if CyaA or cAMP signaling affects ER functions. Despite of not having observed any impairment of the proteasomal processing function, the present data do not allow to conclude definitively at which level the CyaA activity impaired the capacity of LPS-stimulated DCs to activate $CD8^+$ T cells. This issue is currently under investigation and will be subject to a separate study.

B. pertussis virulence factors FHA and CyaA were previously shown to induce IL-10-producing T regulatory cells (Tr1) through immunomodulatory effects on DCs [18,52,53]. Moreover, it has been shown that $CD25^-Foxp3^+$ T regulatory cells are the predominant suppressive subtype in the lungs of *B. pertussis*-infected mice [54]. In this study we observed that CyaA-treated DCs expanded the numbers of $CD4^+CD25^+Foxp3^+$ T regulatory cells *in vitro*. However, it remains to be established whether like for the other types of T regulatory cells [3,52], the $CD4^+CD25^+Foxp3^+$ T cells participate in subversion of *B. pertussis* clearance and/or limit the immune-mediated pathology during *B.* pertussis infection.

cAMP signaling was recently implicated not only in induction of $CD4^+CD25^+Foxp3^+$ T cells but also in the development of Th17 cells [55], which together with Th1 cells, are important for clearance of *B. pertussis* from the respiratory tract of infected mice [9]. We found that CyaA-induced cAMP signaling also enhanced the capacity of TLR-activated DCs to stimulate IL-10 and IL-17 production with limited IFN-γ production also by $CD8^+$ T cells. IL-17-producing $CD8^+$ T cells (Tc17) were, indeed, shown to be involved in modulation of inflammatory immunity during some viral infections [56]. There are, however, no reports yet on the role of Tc17 during *B. pertussis* infection. Although $CD8^+$ T cells were found to be dispensable for resolving *B. pertussis* infection in the mouse model [22,23], in humans IFN-γ producing $CD8^+$ T cell responses were detected in a cohort of *B. pertussis*-infected infants and young adults, as well as in vaccinated children [24]. It remains, hence, to be determined whether these cells are induced by the adjuvant activity of *B. pertussis* virulence factors, or whether $CD8^+$ T cells actively participate in host defense against *B. pertussis*. $CD8^+$ T cells might, indeed, have a role in cytokine production, as well as in cytolytic activity against infected human macrophages, in which *B. pertussis* would survive and grow for some time, as recently documented [57]. Therefore subversion of $CD8^+$ T cells responses by CyaA-treated DCs *in vivo* appears to be a relevant hypothesis to test in future studies.

Supporting Information

Figure S1 Phenotypic analysis of BMDCs and MDDCs. (A) BMDC at day 5 (D5) and day 8 (D8) of culture were analyzed for the expression of H2-k^b, I-A/I-E, CD11c, CD11b, macrophage marker F4/80, granulocyte marker Gr-1 and B cell marker B220 by flow cytometry. DCs at days 7–8 were used for experiments. Histograms are representative of 3 experiments (C57BL/6 mice). The data in graph represent mean values of MFI ± SEM of n = 3. (B) PBMCs at day 0 and MDDCs at day 5 of culture were compared for the expression of CD14, CD1a, CD11c, HLA-DR by flow cytometry. Histograms represent 3 donors showing their variability.
(TIF)

Figure S2 CyaA differentially modulates TLR-induced maturation and cytokine production of DCs. BMDCs or MDDCs were left untreated (medium) or incubated with LPS (100 ng/ml BMDCs or 1 µg/ml MDDCs) or in combination with 10 ng/ml CyaA or CyaA-AC^-. (A) Expression of H-2K^d, I-A/I-E, CD80, CD86, CD40 and CD54 in living $CD11c^+$ BMDCs was determined by flow cytometry after 18 h. Expression of HLA-DR, CD80, CD86, CD40 and CD83 in living $CD11c^+$ MDDCs was determined by flow cytometry after 24 h. Values represent the means ± SEM of n = 4–6 or 5 donors, respectively, where the expression of molecules by LPS-stimulated DCs (LPS) was set as 1.0 (* $p<0.05$). (B) Secretion of IL-10 and IL-12p70 was determined from BMDC culture supernatants by ELISA after 18 h and from MDDC culture supernatants by Luminex after 24 h. Values represent the means ± SEM of n = 4 or 5 donors, respectively.
(TIF)

Figure S3 OVA protein at low concentration is taken up by TLR-stimulated DCs solely via receptor-mediated endocytosis which is unaffected by CyaA. BMDCs left untreated, incubated with LPS (100 ng/ml) alone or in combination with 10 ng/ml of CyaA for 30 min, followed by the incubation with mannan (1 mg/ml) or Poly(I) (10 µM) to block receptor-mediated endocytosis for 30 min. After that OVA-FITC (5 µg/ml) was added to samples for 30 min. The antigen uptake in living $CD11c^+$ cells was determined by flow cytometry. Values represent means ± SEM of n = 4 where OVA-FITC taken up by LPS-treated DC (medium) was set to 100% of MFI.
(TIF)

Figure S4 Analysis of $CD4^+CD25^+Foxp3^+$ T regulatory cells. Gating strategy for mouse (A) and human (B) T regulatory cells is shown.
(TIF)

Figure S5 BMDCs treated with CyaA and LPS induce IL-10 and IL-17-secreting CD4$^+$ T cells with limited IFN-γ production. BMDCs were left untreated, incubated with LPS (100 ng/ml) alone or in combination with CyaA or CyaA-AC$^-$ at 10 ng/ml in the presence of OVA at 2.5 μg/ml for 4 h prior to co-cultivation with naïve CFSE-labeled OT-II CD4$^+$ T cells. After 72 h IL-10, IL-17 and IFN-γ production in DC-CD4$^+$ T cell culture supernatants was determined by ELISA. Values represent means ± SEM of n = 3 (* $p<0.05$).
(TIF)

Figure S6 CyaA induces phenotypic maturation of DCs. (A) MDDCs and BMDCs (2×10^5/sample) were left untreated, incubated with LPS alone or with CyaA or CyaA-AC$^-$ at 10 ng/ml for 24 h at 37°C. The expression of H2-K^b, I-A/I-E or HLA-DR, CD80, CD83, CD40 and CD54 was detected by flow cytometry. The data represent mean values ± SEM of n = 4 or 5 donors (* $p<0.05$). (B) The cytokines in supernatants of cell culture were detected by Luminex (MDDCs) or by antibody array (RayBio; BMDCs). The data represent mean values ± SEM of n = 4 or 5 donors (* $p<0.05$). (C) Mixed lymphocyte reaction: MDDCs were left untreated, incubated with LPS alone or with CyaA or CyaA-AC$^-$ at 10 ng/ml for 24 h at 37°C. Allogeneic CFSE-labeled T lymfocytes were added at T cell: MDDC ratio of 10 : 1. IL-2 (50 U/ml) was added on day 3. On day 4, the proliferation of T cells was determined by flow cytometry. BMDCs were incubated with CyaA or CyaA-AC$^-$ at 10 ng/ml for 24 h at 37°C. The proliferation of allogeneic (BALB/c mice) CFSE-labeled T cells was determined by flow cytometry after 72 h. The histograms are representative and the graphs show mean values ± SEM of n = 4 or 5 donors (* $p<0.05$).
(TIF)

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Author Contributions

Conceived and designed the experiments: IA JK LT RS PJR KHGM MK PS. Performed the experiments: IA JK AK MS JT HJ BC JM LB. Analyzed the data: IA JK AK JM MS MK JT. Contributed reagents/materials/analysis tools: PS KHGM RS LT IA. Contributed to the writing of the manuscript: IA JK PS.

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6.4 PUBLICATION 3

Stanekova, Z., Adkins, I., **Kosova, M.**, Janulikova, J., Sebo, P., & Vareckova, E. (2013). Heterosubtypic protection against influenza A induced by adenylate cyclase toxoids delivering conserved HA2 subunit of hemagglutinin. *Antiviral Res, 97*(1), 24-35. doi:10.1016/j.antiviral.2012.09.008

The influenza virus causes the influenza disease, commonly called as flu. Symptoms of influenza can be mild to severe. Usually the disease manifests itself by a high fever, runny nose, sore throat, muscle and joint pain, severe malaise, cough and headache. The virus is spread easily by coughing or sneezing. Most people recover within one or two weeks without any medical treatment, but in some people it can lead to more severe conditions. Annually, 5%–10% adults and 20%–30% children are infected by influenza, with 3 to 5 million cases of severe illness, causing about 250 000 to 500 000 deaths. In the last century, there were several flu pandemics, the largest of them Spanish influenza in 1918, Asian influenza in 1958, and Hong Kong influenza in 1968. Each resulted in more than a million deaths. There are three types of influenza viruses: A, B and C. The type A is the most virulent of them causing the most severe disease. The influenza A virus can be further divided into different subtypes based on the combination of various virus surface proteins, with H1N1 and H3N2 subtypes being currently most prevalent (www.who.int/ mediacentre/factsheets/fs211/en/).

Influenza vaccines are newly prepared every year according to specific subtypes of influenza viruses which are circulating among population. Hence, these vaccines protect only against vaccine strains and their close antigenic variants. This is quite an ineffective strategy and current research is focused on development of a universal vaccine, which would be able to provide cross-protection against various serotypes of influenza viruses. For that purpose, we have chosen a promising antigen, the HA2 subunit of the hemagglutinin, which is conserved among various hemagglutinin subtypes of influenza A viruses. The capacity of the adenylate cyclase toxoid to penetrate into cell cytosol, together with its potent adjuvant effect can be used for the delivery of inserted antigens for processing and presentation in complexes with MHC molecules on the surfaces of DC and for the induction of T lymphocyte responses. As the adenylate cyclase toxoid has been used to induce

potent immune responses against various viruses, we wanted to test if this could be also the case for the influenza virus. Moreover, the toxoid has been shown to deliver passenger antigens for both MHC class I and II restricted presentation pathways thus stimulating effective $CD4^+$ and $CD8^+$ T lymphocytes responses which both are important for immunity against the influenza infection. We took advantage of this ability of CyaA and designed a novel CyaA construct, a genetically detoxified adenylate toxoid with an inserted segment of HA2 (residues 23 to 185) and tested its ability to induce protective immunity against influenza in mice.

This construct elicited specific Th1 polarized T cell responses against $HA2_{93-102}$, $HA2_{96-104}$ and $HA2_{170-178}$ epitopes. Moreover, it also induced a strong cross-protective HA2-specific antibody response. BALB/c mice that were immunized with such CyaA constructs recovered from influenza infection 2 days earlier then the control mice. Moreover, CyaA-HA2- immunized mice were protected against a lethal challenge with a $2xLD_{50}$ dose of a homologous influenza A virus of the H3 subtype and even against the infection with a heterologous virus of the H7 subtype. Importantly, this is the first report on heterosubtypic protection against influenza A infection mediated by an HA2-based vaccine that can induce both humoral and cellular immune responses without adjuvant.

My contribution: Preparation of recombinant CyaA toxoids carrying influenza epitopes, generation of BMDC, *in vitro* assays testing antigen delivery capacity of toxoids.

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Heterosubtypic protection against influenza A induced by adenylate cyclase toxoids delivering conserved HA2 subunit of hemagglutinin

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ABSTRACT

The protective efficacy of currently available influenza vaccines is restricted to vaccine strains and their close antigenic variants. A new strategy to obtain cross-protection against influenza is based on conserved antigens of influenza A viruses (IAV), which are able to elicit a protective immune response. Here we describe a vaccination approach involving the conserved stem part of hemagglutinin, the HA2 subunit, shared by different HA subtypes of IAV. To increase its immunogenicity, a novel strategy of antigen delivery to antigen presenting cells (APCs) has been used. The HA2 segment (residues 23–185) was inserted into a genetically detoxified adenylate cyclase toxoid (CyaA-E5) which specifically targets and penetrates CD11b-expressing dendritic cells. The CyaA-E5–HA2 toxoid induced $HA2_{93-102}$, $HA2_{96-104}$ and $HA2_{170-178}$-specific and Th1 polarized T-cell responses, and also elicited strong broadly cross-reactive HA2-specific antibody response. BALB/c mice immunized with three doses of purified CyaA-E5–HA2 without any adjuvant recovered from influenza infection 2 days earlier than the control mock-immunized mice. More importantly, immunized mice were protected against a lethal challenge with $2LD_{50}$ dose of a homologous virus (H3 subtype), as well as against the infection with a heterologous (H7 subtype) influenza A virus. This is the first report on heterosubtypic protection against influenza A infection mediated by an HA2-based vaccine that can induce both humoral and cellular immune responses without the need of adjuvant.

1. Introduction

Influenza viruses are highly contagious pathogens that remain a major medical problem and serious threat to human health. The major drawback of current vaccines is that they induce virus-neutralizing (VN) antibodies targeted mostly against the immunodominant HA1 subunit of hemagglutinin (HA), which is highly variable. The VN antibodies prevent infection by blocking virus attachment to the cell surface. However, the narrow specificity of VN antibodies makes current vaccines ineffective against drifted influenza A viruses and against new emerging influenza virus variants with a pandemic potential. Since T-cell immunity plays an important role in clearance of the virus and contributes to the milder course of infection, the design of new vaccines inducing also T cell responses is desirable in order to achieve a broader control of the influenza infection. Vaccines based on the conserved antigens which would elicit both antibody and T cell responses are likely to provide a universal strategy for the control of unexpected flu outbreaks (Wang and Palese, 2009).

The evolution of influenza viruses have resulted in 17 HA subtypes that are further divided into two major phylogenetic groups: group 1 (subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13) and group 2 (subtypes H3, H4, H7, H10, H14 and H15) (Tong et al., 2012; Nabel et Fauci, 2012). Recent studies suggest that the conserved stem region of HA2 glycoprotein (HA2 gp) represents a promising candidate for the preparation of a universal flu vaccine (Gocník et al., 2008; Wang et al., 2010a,b; Steel et al., 2010; Bommakanti et al., 2010). The influenza virus HA is a polypeptide synthesized as a precursor (HA0) that trimerizes in the endoplasmic reticulum and is transported to the cell surface via the Golgi apparatus. The HA0 is post-translationally cleaved by host proteases into two subunits HA1 and HA2, which remain linked by a single

Abbreviations: IAV, influenza A virus; HA, hemagglutinin; HA2, the light chain of hemagglutinin; APCs, antigen presenting cells; CyaA, adenylate cyclase; CyaA-E5, adenylate cyclase toxoid; CyaA-E5–OVA, adenylate cyclase toxoid with inserted OVA epitope; CyaA-E5–HA2, adenylate cyclase toxoid with inserted HA2 epitopes; VN antibodies, virus neutralizing antibodies; CTL, cytotoxic T cells; A/Miss, influenza virus A/Mississippi/1/85 (H3N2); A/Chick, influenza virus A/Chicken Germany/34 (H7N1); LD_{50}, lethal dose of virus at which 50% of infected mice died; DCs, dendritic cells; PBS, phosphate buffered saline; EHA2, ectodomain of HA2 aa 23–185; NP, influenza A nucleoprotein; IFN, interferon.

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disulphide bond (Skehel and Wiley, 2000). Unlike HA1, the HA2 subunit is relatively well conserved among various influenza strains. HA2 is responsible for the fusion of viral and cell membranes in the endosomes, thus allowing the release of a ribonucleoprotein complex of the virus into the cytoplasm (Gerhard et al., 2006). It was shown that HA2-specific antibodies derived from mice (Okuno et al., 1993; Varečková et al., 2003a), humans (Kashyap et al., 2008; Corti et al., 2010) or recombinant libraries (Ekiert et al., 2009; Sui et al., 2009; Throsby et al., 2008; Lim et al, 2008) are broadly neutralizing against group 1 and group 2 of influenza viruses (Varečková et al., 2008; Stropkovská et al., 2009; Wang et al., 2010a,b; Corti et al., 2011). These studies suggest that epitopes in the stem region of HA are accessible to antibodies that can prevent the fusion step and inhibit a viral replication *in vitro* as well as *in vivo*, thus contributing to an earlier recovery from the influenza infection (reviewed in Staneková and Varečková, 2010). Moreover, it was shown that vaccines designed to elicit HA2-specific antibodies confer protection against a lethal influenza infection (Gocník et al., 2007, 2008; Wang et al., 2010b; Steel et al., 2010; Bommakanti et al., 2010).

In the present study, we have exploited a genetically detoxified *Bordetella pertussis* adenylate cyclase toxin (CyaA) for the development of a cross-protective vaccine carrying the stem part of the HA, HA2 gp. The CyaA toxoid has been previously shown to specifically target and penetrate into cytosol of human and animal myeloid phagocytic cells expressing the $\alpha_M\beta_2$ integrin serving as complement receptor 3 (CR3), and known also as CD11b/CD18 or Mac-1 (Guermonprez et al., 2001). This receptor is expressed on professional antigen presenting cells (APCs), such as the myeloid dendritic cells (DC), neutrophils/granulocytes, macrophages, natural killer cells (NK), as well as on a restricted subset of B and NKT lymphocytes (Guermonprez et al., 2001, 2002; Simsova et al., 2004). The capacity of CyaA to selectively penetrate $CD11b^+$ APCs was recently exploited for the development of an antigen delivery platform that enables targeting of passenger antigens into the cytosol of APCs for processing and presentation on MHC class I molecules (www.genticel.com). In parallel, however, a fraction of the cell-bound CyaA molecules is endocytosed with the receptor and reaches the compartment in which processing and loading of antigens onto MHC II molecules occurs. Therefore CyaA-based vaccines could be used for simultaneous induction of strong antigen-specific $CD4^+$ T helper, as well cytotoxic $CD8^+$ T lymphocyte (CTL) responses. CyaA-based vaccines have already been shown to be effective in protecting vaccinated animals against a lethal viral challenge or against growth of transplanted tumors (Sebo et al., 1995; Sakamoto et al., 1992; Guermonprez et al., 1999; Fayolle et al., 1996, 1999; Saron et al., 1997; Loucka et al., 2002; Schlecht et al., 2004; Préville et al., 2005; Berraondo et al., 2007). Furthermore, the use of CyaA vector allowed to induce and also potentiate a long-lasting antigen-specific humoral immune response (Mascarell et al., 2005).

Here we used the genetically detoxified CyaA as a tool for delivery of the conserved stem portion of the HA2 protein (amino acids 23–185) originated from the human H3 subtype virus (A/Aichi/2/68 (H3N2). Vaccination of BALB/c mice with this immunogen elicited both, HA2-specific $CD8^+$ and $CD4^+$ T cell responses, as well as, a strong HA2-specific antibody response, which cross-protected mice against lethal infection with the human-H3 or the highly pathogenic avian-H7 influenza A virus types, respectively.

2. Material and methods

2.1. Viruses

All virus stocks used in this work originated from the collection of viruses of the Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Virus stocks were propagated in 10-day-old embryonated chicken eggs for 48 h at 35 °C. Infectious allantoic fluid was aliquoted and stored at −80 °C. The virus titer was evaluated by micro-hemagglutination assay using guinea pig erythrocytes.

2.2. Purified viruses

A/PR/8/34 (H1N1); A/Beijing/262/95 (H1N1); A/Texas/36/91 (H1N1), A/Dunedin/4/73 (H3N2); A/NT/60/68 (H3N2); A/Mississippi/1/85 (H3N2); Ab4 (H3N2) mutant virus derived from A/Aichi/2/68 (H3N2); A/Wyoming/3/03 (H3N2); A/Sydney/5/97 (H3N2); A/Duck/Czech/56 (H4N6); A/Chicken/Germany/34 (H7N1) were purified from infectious allantoic fluid by sucrose gradient centrifugation as described previously (Russ et al., 1974).

2.3. Preparation of mouse adapted viruses

Mouse adapted human influenza virus A/Mississipi/1/85 (H3N2) (A/Miss) and mouse adapted avian influenza virus A/Chicken/Germany/34 (H7N1) – Rostock Fowl PlagueVirus (A/Chick) were prepared in mouse lungs as follows. Two 6-week old BALB/c mice were infected intranasally under the light anesthesia with 40 μl of allantoic fluid containing wild-type H3N2 or H7N1 virus. Their lungs were harvested 2 days after the infection. Lung cells were homogenized in 1 ml of PBS, pH 7.2. Cell debris was sedimented and supernatant (40 μl) was used for further infection. After six passages, the virus was propagated in 10-day old chicken embryos for 48 or 36 h (for FPV). Infectious dose was estimated by the titration of mouse adapted virus on mice and the median lethal dose (LD_{50}) was determined by the Reed and Muench method. All experiments with highly pathogenic avian influenza virus were conducted under BSL-3 containment, including work with animals.

2.4. Synthetic peptides

H-2Kd-restricted synthetic peptide $CD8^+$ T-cell epitope $HA2_{93-102}$ (SYNAELLVAL), I-Ad restricted $CD4^+$ T-cell epitopes: $HA2_{96-104}$ (AELLVALEN), $CD4^+$ T-cell epitope $HA2_{170-178}$ (RFQIKGVEL) and H-2b restricted synthetic peptide $CD8^+$ T-cell epitope $NP_{366-374}$ (ASNENMETM) were provided by Proimmune (UK).

2.5. Mice

Six week-old BALB/c female mice (Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used in animal experiments. In all experiments presented in this paper, animals were treated according to the European Union standards and the fundamental ethical principles including animal welfare requirements were respected.

2.6. Recombinant HA_{23-185} antigen and CyaA-E5–HA2 toxoids

Free recombinant $HA2_{23-185}$ protein, corresponding to residues 23 to 185 of HA2gp of the recombinant X-31 virus (derived from A/Aichi/2/68 (H3N2) was produced in *Escherichia coli* BL21 cells using the pLM-1 plasmid kindly provided by Prof. D.C. Wiley and Dr. J. Chen, Harvard University, Boston, USA). The HA2 protein was purified as described previously (Chen et al., 1999). The sequence encoding residues 76 to 130 of HA2 was PCR amplified from the plasmid PLM-1, using primers 5′-GACGACGACAAGATGAGAATTCAGGACC TCGAGAAA (forward) and 5′-GAGGAGAAGCCCGGTCAAGCATTTTCCCTCAGTTG (reverse). The amplified DNA was cloned into pTriEx™-4 (Novagen) to generate pTriEx–$HA2_{76-130}$.

For construction of the pT7CTACT-E5–HA2 plasmid, the $HA2_{23-185}$ encoding sequence was amplified from plasmid PLM1

$HA2_{23-185}$ and subcloned into pGem®-T ease vector (Promega) to yield pGem$HA2_{23-185}$ used for HA2 insert preparation. The PCR primers were designed (a) to allow cloning of the $HA2_{23-185}$ insert into a unique *Bsr*GI restriction site located between codons 232 and 233 of the CyaA open reading frame on a pT7CTACT1 vector (Jelinek et al, 2012); (b) to introduce an *Eco*RI restriction site for rapid identification of clones carrying the insert; (c) to introduce stop codons interrupting CyaA-E5–HA2 synthesis when inserted in an inverted orientation and (d) to introduce positively charged flanking amino acid residues towards the termini of the inserted polypeptide sequence, in order to compensate for the negatively charged amino acid residues present at the HA2-processing sites (Karimova et al., 1998). To allow monitoring of the delivery of the AC domain with inserted $HA2_{23-185}$ into the MHC class I pathway, the CyaA-E5–HA2 construct was further tagged by insertion of the $OVA_{257-264}$ epitope SIINFEKL, as described previously (Osicka et al., 2000). The CyaA-E5–HA2 and CyaA-E5–OVA constructs were genetically detoxified by ablating the catalytic adenylate cyclase (AC) enzyme activity by placing a GlySer dipeptide insert between residues 188 and 189, thereby disrupting the ATP binding site of the AC enzyme (Osicka et al., 2000). Orientation and exact sequences of all cloned inserts were verified by DNA sequencing.

The plasmids were transformed into *E. coli* BL21/pMM100 ($lacI^q$) cells and CyaA toxoids were produced and purified by a combination of ion exchange and hydrophobic chromatography (Karimova et al., 1998; Osicka et al., 2000). In the Phenyl-Sepharose chromatography step, the resin with bound CyaA was repeatedly washed with several bed volumes of 60% isopropanol (Franken et al., 2000). This allowed to reduce bacterial endotoxin content below 200 U/mg of the total purified protein, as determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000; Cambrex).

2.7. Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (DCs) were generated according to Lutz et al. (1999). Briefly, DCs were grown in RPMI 1640 medium supplemented with 10% FCS (Life Technologies), 0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 μg/ml amphotericin (Sigma–Aldrich), 50 μM 2-mercaptoethanol, 1% non-essential amino acids (Biochrom), 1 mM sodium pyruvate, 2 mM glutamine and 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). Bone marrow cells (2×10^6), flushed from femurs and tibias of mice, were seeded in 100-mm dishes in 10 ml of medium. During the cultivation, 10 ml of fresh medium was added (day 3) or changed (day 6), respectively. Slightly attached cells were used for experiments at days 6–8. Prior addition of CyaA, the RPMI medium used for cultivation of DC was replaced by Dulbecco's modified Eagle's medium (DMEM medium; 1.9 mM Ca^{2+}) containing 200 U/ml GM-CSF.

2.8. In vitro assay for $OVA_{257-264}$ epitope delivery for MHC class I presentation

Bone marrow-derived DCs were used for *in vitro* presentation of antigens to the B3Z hybridoma $CD8^+$ T cells that recognize the OVA 257–264 peptide SIINFEKL on the murine Kb MHC class I molecule (Karttunen et al., 1992). Presentation of the OVA peptide SIINFEKL on MHC class I K^b molecules on DCs was determined as production of β-galactosidase in B3Z cells, in which T cell receptor binding to SIINFEKL/K^b complex on DCs activated expression of the *lacZ* reporter gene under control of the IL-2 promoter NF-AT elements (Karttunen et al., 1992). DCs were seeded in 96-well plate and incubated with triplicates test samples at various concentrations of CyaA toxoids in DMEM for 4 h at 37 °C in a humid 5% CO_2 atmosphere. The cells were then washed with PBS and further cultured with B3Z hybridoma (10^5 cells/well) in a 200 μl final volume of complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 μM 2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin). After 18 h of incubation at 37 °C, cells were lyzed by addition of 100 μl/well of PBS containing 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% Nonidet P-40 and 0.15 mM chlorophenol red-ß-D-galactopyranoside (CPRG) to determine ß-galactosidase activity. After incubation at 30 °C for 2 h, 50 μl/well of stop buffer (1 M glycine) was added and the absorbance at 570 nm was determined using a microplate reader (Safire², Schoeller Instruments).

2.9. Cell transfection

MDCK cells (5×10^4 cells/well) were grown in 24-well plates to 70–80% confluence. Transfection of plasmid pTriEx–$HA2_{76-130}$ was performed using TurboFect™ *in vitro* Transfection Reagent (Fermentas) according to the manufacturer's instruction and after 24 h post-transfection (p.t.), the samples were used in further experiment.

2.10. Immunization and challenge of mice

Six week-old female BALB/c mice were immunized intraperitoneally (i.p) on days 0, 20, 40 with 50 μg CyaA-E5–$HA2_{23-185}$ or CyaA-E5–OVA (control group) in phosphate-buffered saline (PBS). Mice were challenged intranasally (i.n.) on day 60 with a lethal dose of homologous A/Miss ($2LD_{50}$) or heterologous A/Chick ($2LD_{50}$) influenza viruses in a volume of 40 μl. Mice were monitored daily for clinical symptoms and for mortality. All challenge experiments with the highly pathogenic avian A/Chick influenza virus were conducted at an animal biosafety level 3 facility.

2.11. Sera collection

Blood was collected from facial vein of 5 mice of each experimental group before the first immunization and after each immunization dose.

2.12. Analysis of antibody responses by indirect ELISA

Individual mice sera were tested for the presence of HA2-specific antibodies by enzyme-linked immunosorbent assay (ELISA). In brief, 96-well microplates were coated overnight with EHA2 (30 ng/100 μl) or purified influenza viruses (300 ng/100 μl) in PBS at 4 °C. Before adding serum samples the adsorbed purified virus was treated with buffer pH 5 or pH 7 for 30 min. Serum samples in 2-fold dilutions in PBS containing 0.5% ovalbumin (starting from dilution 1:100) were added to coated plate wells for 90 min of incubation at room temperature and the microplates were repeatedly washed with PBS containing 0.02% Tween-20, before horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a sera (AbCam®, USA) were added, followed by addition of o-phenylenediamine with 0.03% hydrogen peroxide (Sigma) as substrate. The reaction was stopped by adition of 3 M HCl and absorbance at 492 nm was measured in a Multi-Mode Microplate ELISA Reader (BioTek, Synergy HT). The titers of specific antibodies were calculated as the reciprocal values of serum dilution at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. Cut-off was estimated as the average value of 3 preimmune serum samples ± 3 standard deviations.

2.13. Indirect immunofluorescence

MDCK cells were transfected with plasmid DNA using Turbofect (Fermentas) according to the manufacturer's protocol. In brief,

MDCK cells were grown on glass cover slips to 60–70% confluence. Twenty four hours after transfection the cells were fixed with 3% paraformaldehyde (Sigma) in PBS for 10 min and permeabilized with 1% Triton X-100 (Koch-Light) for 60 s. Cell samples were washed three times with PBS, incubated with the mixture of the primary antibodies for 1 h at room temperature, washed repeatedly and incubated for 1 h with anti-mouse IgG antibody conjugated to FITC (Dako). After the final wash, the cell samples were labeled with DAPI (4′,6-diamino-2-phenylindole) in mounting medium (Santa Cruz Biotechnologies). Fluorescence of transfected cells was evaluated in a fluorescent microscope (Leica CTR 600).

2.14. Microneutralization assay

Virus (A/Miss-150 pfu/well or Ab4-250 pfu/well) were incubated with preimmune or immune serum from mice immunized with CyaA-E5–HA2 or CyaA-E5–OVA (final dilution 1:100) at 37 °C/30 min. Monolayers of MDCK cells in 96-well microplate were infected with the mixture of virus–serum (100 μl/well) and incubated at 37 °C for 18 h. After the incubation, cells were fixed with cold methanol and replicated virus was detected with a nucleoprotein-specific monoclonal antibody 107L (1.5 μg/ml) (Varečková et al., 1995) and goat anti-mouse IgG conjugated with a horseradish peroxidase (Bio-Rad). After adding of substrate solution containing 3-amino-9-ethylcarbazole (Sigma, A6926) with 0.03% H_2O_2, red stained infected cells were evaluated. The antibody-mediated inhibition of virus replication was estimated as the reduction of number of infected cells per well by serum antibodies of immunized and control group of mice.

2.15. Estimation of infectious virus titer in mouse lungs

Lungs of immunized mice challenged with H3N2 (A/Miss) or H7N1 (A/Chick) were collected in two day-intervals (lungs from 2 mice/group for each interval) and viral load was measured. Lungs were homogenized in 1 ml of PBS and the titer of the infectious virus in lung suspension was estimated by a rapid culture assay on MDCK cell monolayer in the presence of trypsin, as described previously (Tkáčová et al., 1997). Briefly, a confluent MDCK cell monolayer grown in a 96-well microtitre plate was infected with 2-fold dilutions (in PBS, pH 7.2) of lung homogenate (100 μl/well) and incubated for 45 min at room temperature. Cells were then incubated for 18 h in serum-free Ultra-MDCK medium containing 0.5 μg TPCK trypsin (Sigma) at 37 °C in 5% CO_2 atmosphere. After 18 h the cells were fixed with methanol and the virus was visualized using a sandwich of the monoclonal antibody 107L (1.5 μg/ml), specific for nucleoprotein of influenza A virus (Varečková et al., 1995), using a horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) and substrate solution containing 3-amino-9-ethylcarbazole (Sigma, A6926) with 0.03% H_2O_2. Infected cells were scored positive as differentiated visibly red stained cells by light microscopy. The titer of infectious virus was determined as the reciprocal value of the highest dilution of the tested lung homogenate at which infected cells were still detected.

2.16. Detection of vRNA in lungs of infected mice

Viral RNA (vRNA) in mouse lungs was detected by RT-PCR. Total RNA from lung-cell homogenate (200 μl) was isolated with the RNA Instapure System (Eurogentec) according to manufacturer's instructions. The final RNA precipitate was resuspended in 20 μl RNase/DNase-free H_2O supplemented with 2 U RNase inhibitor (Fermentas) μl^{-1}. The reaction mixture for reverse transcription (60 min at 42 °C) contained 5 μl total RNA, 1 mM each dNTP, 5× RT reaction buffer (Fermentas), 0.2 μg random heptamer (Invitrogen), 200 U Moloney murine leukemia virus reverse transcriptase (Fermentas) and H_2O up to a final volume of 20 μl. Amplification of cDNA was carried out using oligonucleotide primers specific for the influenza A nucleoprotein (NP). Primers specific for NP (H3 subtype) 5′-GTGAGGATGCAACAGCTGGTCTAAC (forward) 5′-TACCCCTCTTTTTCGAAGTCGT AC (reverse) and NP (H7 subtype) 5′-ATCCATCATTGCTCGTTGTG (forward), 5′-GCATCTGTTGGGAGAATGGT (reverse) were designed. The β-actin primers 5′-AGGTCATCACTATTGGCAAC (forward) and 5′-ATCTTGATCTTCATGGTGCT (reverse) were used as normalization control.

2.17. IFN-γ ELISPOT assay

Interferon (IFN-γ) ELISPOT was performed using the Elispot kit (eBioscience) according to manufacturer's instructions. Briefly, mice were immunized i.p. with 50 μg CyaA-E5 –$HA2_{23–185}$ or CyaA-E5–OVA in PBS. Spleens were removed at day 9 after the first immunization (three mice per group) and mechanically dissociated into single cell suspensions by 70 μm cell strainers (Falcon). The splenocyte suspensions were stimulated in the presence of the 10 μM synthetic peptide ($HA2_{93–102}$, $HA2_{96–104}$, $HA2_{170–178}$) in 96-multiscreen filtration plates coated with anti-mouse IFN-γ antibody for 24 h at 37 °C and in 5% CO_2 in RPMI media containing 10% FCS, antibiotics penicillin (100 U/ml) and streptomycin (100 mg/ml), as described previously (Jackson et al., 1994; Saikh et al., 1995). IFN-γ secreting cells were detected with a sandwich of biotinylated anti-mouse IFN-γ antibody with streptavidin peroxidase using hydrogen peroxide and 3-amino-9-ethylcarbazole (Sigma) as substrates in 0.1 M sodium acetate (pH 5.0). The numbers of spot-forming cells (SCF) per well were counted with the aid of a dissecting microscope and expressed as the mean number of triplicates of IFN-γ SCF per 2×10^6 cells with the standard deviation. Two negative controls for splenocytes from mice immunized with CyaA-E5–$HA2_{23–185}$ were used. Cells were incubated with irrelevant peptide $NP_{366–374}$ (C57BL6, H-2^b) as well as without any peptide. As another negative control splenocytes from mice immunized with CyaA-E5–OVA were incubated with relevant or irrelevant peptide, or without any peptide, respectively.

3. Results

3.1. CyaA toxoid delivers the inserted OVA-tagged $HA2_{23–185}$ peptide into cytosol of antigen presenting cells

To enable targeting of the $HA2_{23–185}$ antigen into cytosol of CD11b-expressing antigen presenting cells (APCs) for processing and subsequent presentation on MHC class I molecules to $CD8^+$ T cells, we inserted an $OVA_{257–264}$ epitope-tagged $HA2_{23–185}$ polypeptide segment between the residues 232 and 233 of the AC domain of genetically detoxified CyaA. The resulting CyaA-E5–HA2 toxoid and its mock CyaA-E5–OVA variant were produced in *E. coli* BL21/pMM100 cells and purified close to homogeneity (not shown). Prior the use for immunization of mice, the capacity of toxoids to bind APCs and to deliver the antigenic cargo into their cytosol for processing and presentation was assessed *in vitro*. It has been repeatedly shown that only toxoids capable of translocating their OVA epitope-tagged AC domain into cytosol of APCs elicit a SIINFEKL peptide-specific stimulation of co-incubated B3Z $CD8^+$ T hybridoma cells that recognize the epitope in complex with K^b MHC I molecules on APCs (Holubova et al., 2012).

As shown in Fig. 1, DC were incubated with the increasing concentrations of CyaA-E5–HA2 or CyaA-E5–OVA toxoids for 4 h before being mixed with the $OVA_{257–264}$-specific $CD8^+$ B3Z hybridoma T cells. Starting from a 5 nM toxoid concentration (1 μg/ml), a comparable efficiency of $OVA_{257–264}$ epitope (SIINFEKL) presentation *in vitro* on DC to B3Z T cells was detected for

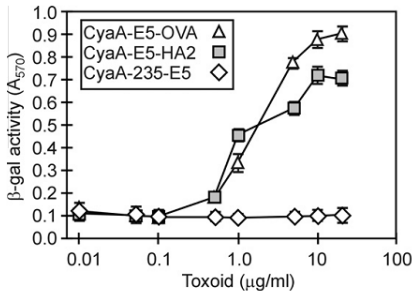

Fig. 1. Antigen delivery capacity of the CyaA-E5–HA2 construct *in vitro*. Bone marrow-derived dendritic cells were incubated with indicated concentrations of the CyaA-E5–HA2 and CyaA-E5–OVA toxoids for 4 h before the B3Z CD8$^+$ T hybridoma cells (K^b restricted) were added and incubation was continued for 18 h. Stimulation of B3Z cells by the SIINFEKL epitope of ovalbumin presented on K^b MHC I molecules was measured as the amount of β-galactosidase enzyme accumulated in B3Z cells (Karttunen et al., 1992). Mock CyaA-233-E5 toxoid was used as a negative control (CyaA-233-E5) 1 μg/ml corresponds to 5 nM toxoid concentration.

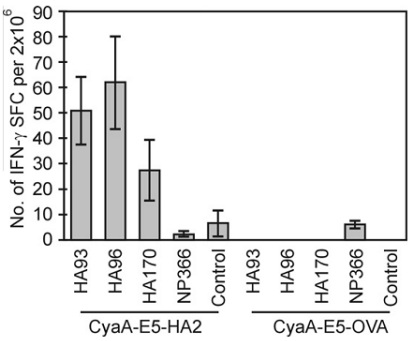

Fig. 2. Immunization with CyaA-E5–HA2 induces antigen-specific T cell response. Three BALB/c mice per group were immunized i.p. with a single dose of 50 μg of CyaA-E5–HA2 or of CyaA-E5–OVA (negative control). The presence of HA2-specific IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells in mouse spleens was assessed on day 9 after immunization by ELISPOT assay upon *in vitro* restimulation with the indicated peptides. Numbers of IFN-γ-secreting cells represent the average values plus standard deviations from the pooled triplicate values from two independent experiments (n = 6). Three types of negative controls were used: (i) "Control" means that cells were not stimulated with any peptide, (ii) NP366 means that corresponding peptide was used as an irrelevant peptide, (iii) splenocytes from mice immunized with CyaA-E5–OVA incubated with relevant or irrelevant peptides as well as cells not stimulated with any peptide, all represent further negative controls. Baseline corresponds to a negative reaction (no spots).

both CyaA-E5–HA2 or CyaA-E5–OVA toxoids, when assessed as an IL-2-dependent expression of ß-galactosidase in stimulated B3Z cells. In contrast, no unspecific B3Z stimulation was observed with DC incubated with the control CyaA-233-E5 toxoid lacking the $OVA_{257–264}$ epitope. These results show that the insertion of the 170 residue long $OVA_{257–264}$-tagged $HA2_{23–185}$ antigen polypeptide did not greatly affect the capacity of the resulting CyaA-E5–HA2 toxoid to translocate its AC domain into APC cytosol for processing by proteasome and subsequent presentation of the delivered $OVA_{257–264}$ epitope on K^b MHC class I molecules.

3.2. CyaA-E5–HA2 induces HA2-specific T and B cell responses in BALB/c mice

The $HA2_{23–185}$ antigenic peptide of the H3 subtype comprises several conserved T and B-cell epitopes of IAV. Therefore, we evaluated its capacity to induce specific T and B cell immune responses in mice when delivered in a form of the CyA-E5–$HA2_{23–185}$ toxoid. BALB/c mice were immunized i.p. with 50 μg of CyaA-E5–HA2 or the mock CyaA-E5–OVA toxoid in PBS without any adjuvant. Induction of HA2-specific CD8$^+$ and CD4$^+$ T cell immune responses was analyzed by IFN-γ ELISPOT assays using splenocytes of mice sacrificed 9 days post immunization. For the detection of specific responses splenocytes were restimulated *in vitro* with synthetic peptides corresponding to the CD8$^+$ T cell *H-2d*-restricted peptide $HA_{93–102}$ (SYNAELLVAL), the CD4$^+$ T cell *I-Ad*-restricted peptides $HA_{170–178}$ (RFQIKGVEL) and $HA_{96–104}$ (AELLVALEN) of HA2, respectively, as these epitopes are known to be cross-reactive among IAV subtypes (Saikh et al., 1995; Jackson et al., 1994). The specificity of stimulation was verified by the use of an irrelevant H-*2b*-restricted peptide of the $NP_{366–374}$ epitope (ASNENMETM). As shown in Fig. 2, *in vitro* restimulation with the specific peptides corresponding to the CD8$^+$ T cell epitope $HA_{93–102}$ and CD4$^+$ T cell epitopes $HA_{96–104}$ and $HA_{170–178}$ elicited a specific IFN-γ production by T cells that were present in splenocyte suspensions from CyaA-E5–HA2-immunized mice. No IFN-γ production was detected after restimulation of splenocytes from the control group of mice immunized with CyaA-E5–OVA.

CyaA toxoid has been previously shown to deliver epitopes via endocytosis into the endosomal compartment for MHC class II-restricted presentation to CD4$^+$ T helper cells, which subsequently also increased the specific antibody response (Loucka et al., 2002; Mascarell et al., 2005). Therefore, we further examined whether HA2-specific antibodies were effectively induced by the immunization of mice with the CyaA-E5–HA2 toxoid. Mice were immunized i.p. at days 0, 20 and 40 with 50 μg of CyaA-E5–HA2 in PBS without adjuvant and the control group received CyaA-E5–OVA (50 μg). Another group of mice was immunized i.p. at the same intervals with 5 μg of free $HA2_{23–185}$ protein, which on a molar basis equaled the amount of antigen delivered by the 10-times higher amount of the approximately 10-times larger CyaA-E5–HA2 toxoid (163 residues of $HA2_{23–185}$ compared to the 1898 residue-long CyaA-E5–HA2). The presence of HA2-specific IgG antibodies in the sera of immunized mice was then analyzes by ELISA in serum samples after each immunization. As shown in Fig 3, no anti-HA2 antibodies were detected after the first i.p. immunization (priming), while an about 11-fold higher titer of HA2-specific antibodies was detected after the first booster immunization in the sera of mice immunized with CyaA-E5–HA2, as compared to the sera of mice that received only a HA2 protein. The antibody response was strongly enhanced after the second booster immunization, where an HA2-antibody titer of 71,593 was determined for the sera of mice receiving CyaA-E5–HA, as compared to the titer value of 42,378 for the sera of mice immunized with 5 μg of a purified HA2 protein (Fig 3). Antibody titer was calculated on the basis of reactivity of tested sera in ELISA binding test as described in methods. It represents a reciprocal value of serum dilution, at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. These results showed that delivery of $HA2_{23–185}$ by the CyaA-E5–HA2 toxoid vector also enhanced the specific antibody response against $HA2_{23–185}$, as compared to that induced by a protein antigen only.

As shown in Fig. 4, immunization with CyaA-E5–HA2 resulted in the induction of antibodies of qualitatively different isotype composition when compared with the antibody composition induced by a HA2 protein alone. CyaA-E5–HA2 induced simultaneous production of IgG1 (56.6%), IgG2a (35%) and IgG2b (8.4%) antibody isotype responses against $HA2_{23–185}$, suggesting that a mixed Th1 and Th2-polarized immune response was induced. In

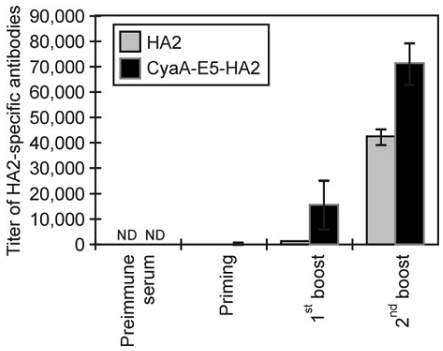

Fig. 3. Immunization with CyaA-E5–HA2 elicits anti-HA2 antibody response. Five BALB/c mice per group were immunized at days 0, 20, and 40 i.p. with 50 μg of CyaA-E5–HA2 or CyaA-E5–OVA, or with 5 μg of purified HA2 protein in PBS without adjuvant. HA2-specific IgG antibody titers were determined in sera of mice after each immunization step, i.e. at days 19 (priming), 39 (first boost), and 59 (second boost). Titer of antibodies specific to HA2 gp (*Y* axis) represents the reciprocal value of serum dilution at the point where the regression line drawn through the three points of the titration curve crossed the cut-off value. Cut-off was estimated as the average value of 3 preimmune serum samples ± 3 standard deviations. *Note:* ND means not detectable level of antibodies.

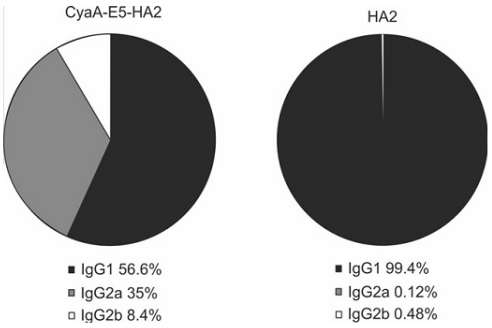

Fig. 4. Immunization with CyaA-E5–HA2 induces a mixed IgG isotype profile of HA2-specific antibodies. BALB/c mice (5 per group) were immunized i.p. with 50 μg of CyA-E5–HA2 or with 5 μg of purified HA2 protein and blood was drawn from mice 14 days after the second immunization boost (i.e. at day 59 of immunization). Individual sera were analyzed for titers of HA2-specific antibodies of the IgG1, IgG2a or IgG2b isotypes by ELISA.

contrast, nearly 100% IgG1 antibody response was detected in the sera of mice immunized i.p. with a HA2 protein alone, which indicates Th2 polarization.

3.3. Immunization with CyaA-E5–HA2 elicits broadly cross-reactive antibodies

It was important to assess how broadly cross-reactive was the antibody response elicited by CyaA-E5–HA2. The reactivity of antibodies induced after the second booster immunization with influenza A viruses of different HA subtypes as antigens was therefore assessed by ELISA. HA2-specific antibodies induced by CyaA-E5–HA2 (Fig. 5) were cross-reactive with influenza A viruses of the H3, H4 and H7 subtypes, albeit the determined antibody titers against H4 (titer 10.7 ± 1.2 $\log_2$) and H7 (titer: 11.1 ± 1.8 $\log_2$) subtypes were several-fold lower than those against the best recog-

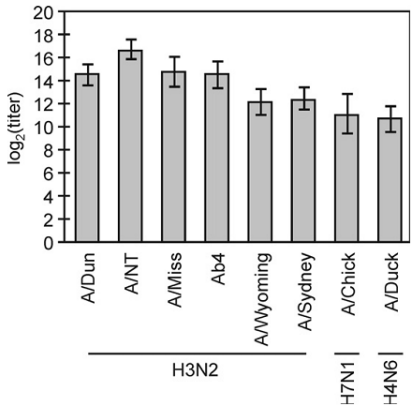

Fig. 5. Immunization with CyaA-E5–HA2 induces IAV subtype cross-reactive HA2-specific antibodies. Titers of HA2-specific antibodies reactive with pH5-treated purified influenza viruses of the indicated subtypes were determined by ELISA. Three individual sera were analyzed for each group of mice after the second booster immunization (day 59). Titer of non-immune serum represents baseline (0). There was not statistically significant difference in the reactivity among IAV of H3 and H7 subtypes in relation to the homologous strain A/Miss (A/Dun $p = 0.9284$, A/NT $p = 0.1378$, Ab4 $p = 0.8509$, Wyoming $p = 0.1014$, Sydney $p = 0.1117$, H7 subtype FPV-R $p = 0.0662$). Significant difference of reactivity in relation to A/Miss was observed with IAV of H4 subtype A/Duck ($p = 0.0290$).

nized A/NT H3N2 virus (16.7 ± 0.8 $\log_2$). No significant differences in reactivity were detected among viruses of H3 and H7 subtypes. However, CyaA-E5–HA2 induced antibodies preferentially bound to pH5 form of HA (Fig. 6). Lower antibody binding was measured with prefusion form of HA, i.e. with the native conformation of HA, though the cross-reactivity pattern of the reactivity was similar to that of pH 5 form.

3.4. CyaA-E5–HA2-induced antibodies recognize the broadly protective $HA2_{76–130}$ region

To corroborate the analysis, we examined whether the induced antibodies can recognize the $HA2_{76–130}$ fragment consisting of residues 76 to 130, which was recently described as a target of the broadly neutralizing 12D1 monoclonal antibody (Wang et al., 2010a,b). Towards this aim, the $HA2_{76–130}$ polypeptide was expressed in pTriEx–HA2-transfected MDCK cells and its recognition by sera from three mice after the 2nd booster immunization with CyaA-E5–HA2 or CyaA-E5–OVA (control) were evaluated by immunofluorescence. As documented in Fig. 7, the serum from CyaA-E5–HA2-immunized mouse showed a positive staining of MDCK cells transfected with pTriEx–HA2, while no reaction was observed with the preimmune or control sera of mice immunized with CyaA-E5–OVA, respectively. Therefore, it can be concluded that the antibody response induced with CyaA-E5–HA2 comprised antibodies specific to the $HA2_{76–130}$ region. Even though this has not been tested, we cannot exclude that also a population of neutralizing antibodies of the 12D1 like specificity was induced.

3.5. Vaccination with CyaA-E5–HA2 accelerates virus elimination from lungs of mice infected with H3 and H7 influenza viruses

To investigate the cross-protective potential of the vaccination with CyaA-E5–HA2, BALB/c mice were immunized with CyaA-E5–HA2 as described above and challenged with a $2LD_{50}$ dose of homologous A/Miss (H3) or heterologous A/Chick (H7) virus sub-

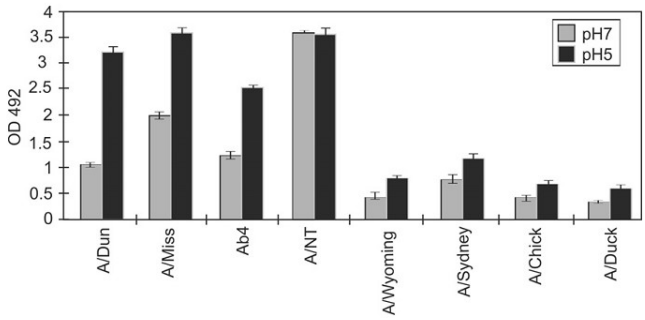

Fig. 6. Reactivity of sera with viruses of native and low pH conformation of HA by ELISA. Purified viruses of appropriate subtypes were used as antigens in native or in pH5 treated form for detection of specific antibodies. The values are corrected for reactivity with non-immune serum.

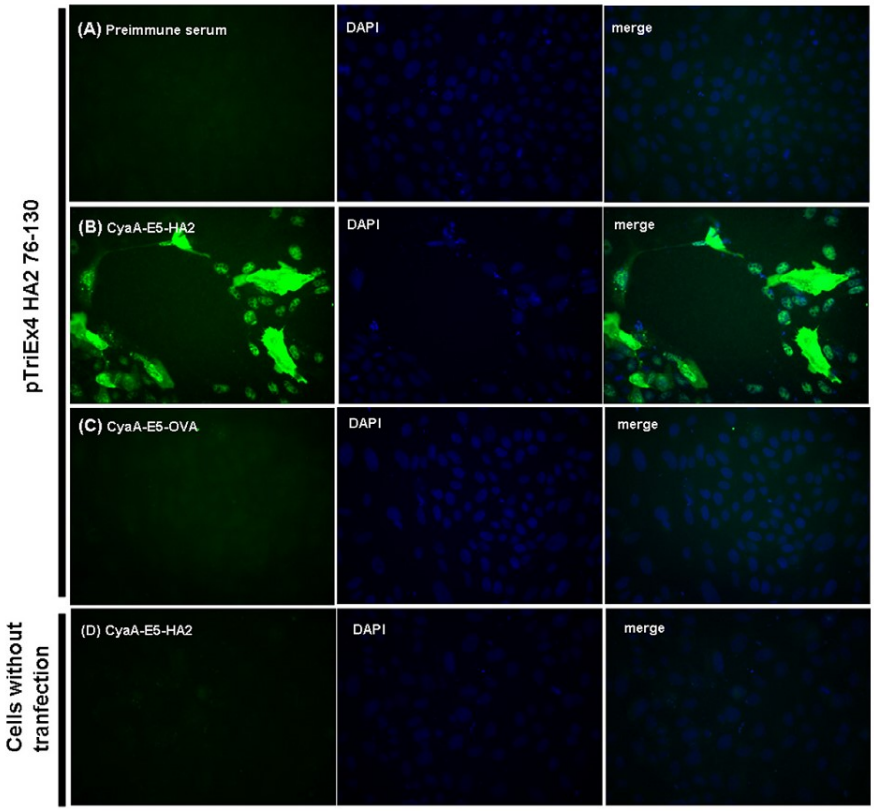

Fig. 7. CyaA-E5–HA2-induced antibodies recognize the conserved $HA2_{76-130}$ part of HA2. MDCK cells were transfected with pTriEx–$HA2_{76-130}$ (A-C) or with mock pTriEx plasmid (D). Cells were stained with preimmune mouse serum (A), with immune serum taken after the second booster immunization with CyaA-E5–HA2 (B) and a control serum of mice immunized with the mock toxoid CyaA-E5–OVA (C), respectively. MDCK cells transfected with empty pTriEx plasmid (D) were used as control for unspecific background staining with immune serum of mice immunized with CyaA-E5–HA2.

types, respectively. The course of the infection, i.e. survival, as well as virus titers in the lungs were followed on days 2, 4, 6, 8, and 10 after infection and compared with mock-immunized mice that received CyaA-E5–OVA or PBS, only. As shown in Fig. 8a, the viral

titers in lungs of CyaA-E5–HA2-immunized mice, challenged with homologous A/Miss virus, reached the maximum ($17 \pm 0.5 \log_2$ units) on day 4 and decreased to $10.6 \pm 1.0 \log_2$ units on day 6, with no infectious virus or vRNA being detectable already on day 8 post infection. In contrast, infectious virus and vRNA were still detected on day 8 post infection in lungs of the control mice that were mock-immunized with CyaA-E5–OVA.

When mice were challenged with the heterologous mouse-adapted A/Chick virus, the viral titers in lungs of CyaA-E5–HA2 immunized mice reached a maximum on the day 2 and were progressively decreasing. On the day 8 p.i., the virus could not be detected anymore. The similar course of the infection has been observed in the control group of mice (Fig. 8b). However, the A/Chick virus vRNA was detected 8 days p.i. in the lungs of both vaccinated and control mice, while the last detectable viral titer determined on the 6th day p.i. was lower in lungs of immunized mice ($6.6 \log_2$ units) than in lungs of control mice ($9.9 \log_2$ units), in correlation with the intensity of corresponding vRNA bands detected by RT-PCR.

These results showed that immunization with CyaA-E5–HA2 enabled accelerated clearance of influenza virus from the lungs of mice infected with the homologous A/Miss virus and resulted in lower infectious titers of the heterologous A/Chick influenza A virus.

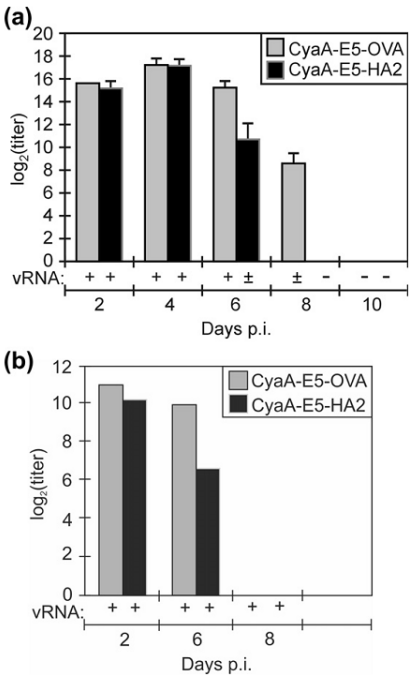

Fig. 8. Immunization with CyaA-E5–HA2 accelerates the drop of vRNA load and clearance of influenza virus in lungs of mice infected with A/Miss (H3N2) virus. Upon i.p. immunization with 3 doses of 50 μg of CyaA-E5–HA2 or CyaA-E5–OVA toxoids, two mice per group were infected on day 59 with a $2LD_{50}$ dose of A/Miss (H3N2) (a) or A/Chick (H7N1) (b) influenza virus. Viral titers and vRNA loads were determined in homogenates of mice lungs at indicated dates post infection by rapid culture and by RT-PCR assays. The titer of infectious virus was determined as the reciprocal value of the highest dilution of sample (lung homogenate) that still infected cells. vRNA was detected in lung homogenates using primers specific for influenza A virus NP. Means of duplicate values from two experiments are given. For H7N1 virus, lungs from only one mouse in each interval was analyzed.

3.6. Immunization with CyaA-E5–HA2 confers cross-protection against a lethal infection of mice with IAV of H3 and of H7 subtypes

To examine the degree of protection resulting from vaccination with the CyaA-E5–HA2 toxoid, we followed the survival of the immunized mice that were challenged on day 60 with a $2LD_{50}$ of homologous (A/Miss, H3N2), or the heterologous (highly pathogenic) avian influenza virus (A/Chick, H7N1). Immunization with the CyaA-E5–HA2 conferred a full protection and 100% of survival of mice against a lethal challenge with A/Miss (H3N2) influenza virus when compared to mock-immunized mice treated with CyaA-E5–OVA ($p = 0.0006$) or PBS-treated mice that all died by the day 10 after infection ($p = 0$) (Fig. 9a). Moreover, the immunization with CyaA-E5–HA2 conferred on mice also a very high level of protection (80%, $p = 0.0007$) against a lethal challenge with the highly pathogenic heterologous A/Chick (H7N1) virus that killed all mock-immunized mice (Fig. 9b). Furthermore, in both challenge studies the course of infection was noticeably milder in the CyaA-E5–HA2-vaccinated mice than in the control group, as judged also from monitoring of clinical symptoms, such as a low activity and an appearance of scrubby fur.

Based on these results it can be concluded that the immunization with CyaA-E5–HA2 conferred an inter-subtype protective immunity against a lethal infection with influenza A viruses. In our previous *in vivo* experiments, mice were fully protected against the homologous virus after the immunization with purified EHA2 applied with Freund adjuvants. However, only a partial and statistically non-significant protection from the infection with the virus of heterologous H7 subtype was achieved (Janulíková et al., 2012). The immunization of mice with EHA2 protein increased the survival of experimental animals from 30% in the control non-immunized mice, to 56%. More effective cross-protection after the immunization with CyaA-E5–HA2 is likely to be due to induction of specific T-cells, which recognize conserved regions on HA2 gp (Saikh et al., 1995; Jackson et al., 1994). Furthermore, it should be noted that cross-protection was achieved by immunization with CyaA-E5–HA2 without the need of any added adjuvant. This suggests that the CyaA toxoid exhibited an adjuvanting capacity itself and can be used for enhancement of HA2 immunogenicity.

3.7. CyaA-E5–HA2 induced antibodies reduce the virus replication

To understand the role of antibodies induced after immunization of mice with CyaA-E5–HA2, their activity was examined in microneutralization test. The cca 50% reduction of virus replication was observed in RCA (Fig. 10) with homologous A/Miss virus as well as with heterologous virus Ab4 of conformationally changed HA of the same H3 subtype.

4. Discussion

We report here that vaccination of mice with a conserved stem part of HA2 of influneza A virus delivered by the *B. pertussis* adenylate cyclase toxoid (CyaA) has a potential for development of HA2-based cross-protective influenza vaccines. This observation is of a particular interest in the light of the currently ongoing phase I clinical trials with cGMP batches of CyaA toxoids that carry the human papillomavirus 16 and 18 antigen E7, aimed for tumor immunotherapy (www.genticel.com), thus paving the way to clinical use of the CyaA-based antigen delivery approach.

One of the drawbacks of currently available influenza vaccines is their incapacity to induce a broadly cross-protective humoral

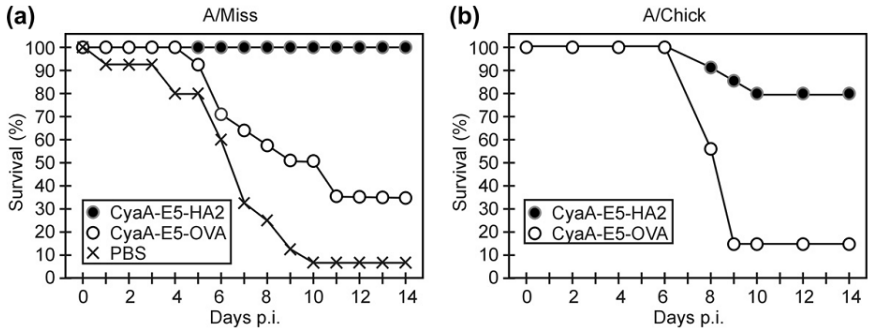

Fig. 9. Immunization with CyaA-E5–HA2 fully protects mice against lethal challenge with the homologous A/Miss (H3N2) virus and confers high level of protection against heterologous challenge with A/Chick (H7N1) virus. Groups of 14 to 15 immunized, or control mice were infected on day 59 with $2LD_{50}$ dose of (a) A/Miss (H3N2) or (b) of A/Chick (H7N1) virus and mice survival was monitored until 14 days post infection. The significance of survival was evaluated using Fisher's exact test. For mice infected with A/Miss (H3N2) the difference in survival between mice treated with CyaA-E5–HA2 ($n = 14$) and CyaA-E5–OVA ($n = 14$) was at the $p = 0.0006$ level. The difference in survival between mice immunized with CyaA-E5–HA2 and control group that received PBS only was at $p = 0.0000$. For mice infected with A/Chick (H7N1) virus the difference in survival between mice immunized with CyaA-E5–HA2 and CyaA-E5–OVA mock-immunized mice was at $p = 0.0007$.

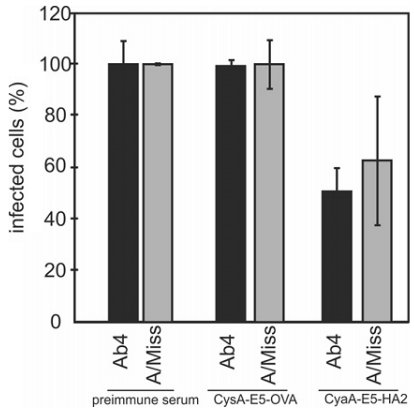

Fig. 10. Antibodies induced with CyaA-E5–HA2 reduce virus replication. MDCK cells were infected with homologous virus A/Miss (H3N2) or with Ab4 virus with conformationally different HA of the same subtype. Axis *Y* represents the percent of infected cells in the presence of preimmune (100%) and HA2-specific serum at dilution 1:100.

and cell-mediated immune response against a variety of influenza A virus subtypes. Several studies have shown that the conserved part of hemagglutinin, its HA2gp stalk, is a promising candidate for development of broadly-protective influenza vaccines (Gocník et al., 2008; Steel et al., 2010; Bommakanti et al., 2010; Wang et al., 2010b). It was observed that HA2-specific antibodies can reduce replication of influenza virus (Lim et al., 2008; Varečková et al., 2003b; Edwards and Dimmock, 2000, 2001; Okuno et al., 1993), improve the recovery from influenza infection and provide protection against a lethal influenza infection (Varečková et al., 2003b; Gocník et al., 2007, 2008; Prabhu et al., 2009). Recently, broadly neutralizing antibodies specific to the HA2 stem of HA were identified, which exhibited activity against both, group 1 and group 2 influenza viruses (Kashyap et al., 2008; Throsby et al., 2008; Sui et al., 2009; Wei et al., 2010; Wang et al., 2010a,b, 2011; Stropkovská et al., 2010; Nabel and Fauci, 2010; Ekiert et al., 2011). These antibodies could be used as a therapeutic cocktail for vaccination against influenza A viruses of different HA subtypes and represent thereby the inspiration for new immunization strategies that would efficiently elicit HA2-specific antibodies.

By fusing the conserved HA2 stalk region segment to a carrier targeting antigen-presenting cells, we developed here an experimental influenza vaccine that exhibited an enhanced cross-protective potential and immunogenicity of HA2 for efficient stimulation of both humoral and cellular immunity. These results go well with previous reports on the use of the adenylate cyclase toxoid as an antigen delivery tool used for induction of both humoral (Mascarell et al., 2005) and cell-mediated immune responses (Guermonprez et al., 1999; Fayolle et al., 1996; Loucka et al., 2002; Schlecht et al., 2004). Of particular interest appears to be the fact that no adjuvant was needed to achieve efficient immunization with CyaA-E5–HA2 that elicited high levels of HA2-specific antibodies and protection against viral challenge up to $6LD_{50}$. This is of particular importance in the light of the quite limited choice of adjuvants approved for use in humans and the recent controversial reports on potential negative side effects of adjuvants used in some commercial flu vaccines (Stropkovská et al., 2010).

Moreover, we have also observed that HA2-specific antibodies induced by the vaccination with CyaA-E5–HA2 were cross-reactive within group 2 HA proteins (H3, H4, H7). However, their reactivity with subtypes of H1 belonging to group 1 was weak (data not shown). Our results showed further that the immunization with CyaA-E5–HA2 induced also antibodies recognizing $HA2_{76-130}$ which might exhibit a similar specificity as the antibody 12D1 that was recently described as broadly neutralizing (Wang et al., 2010a,b). In addition, the vaccination with a synthetic peptide corresponding to residues 76 to 130 of HA2 (the binding region of 12D1) was reported to provide a protection in mice against influenza viruses of phylogenetically distinct subtypes (Wang et al., 2010a,b). This goes well with the here-observed cross-protective potential of the vaccination with CyaA-E5–HA2.

It has been previously shown that the vaccination with adenylate cyclase toxoid-based vaccines allows the induction of Th1-polarized immune responses (Dadaglio et al., 2000; Mascarell et al., 2005). Analysis of anti-HA2 specific immunoglobulin isotypes indicated that i.p immunization with CyaA-E5–HA2 induced a mixed Th1 and Th2 type of immune response. In contrast, prevailing Th2-polarized immune response (Abs of IgG1 isotype), with negligible amount (0.6%) of IgG2a and IgG2b isotypes, was

obtained uppon the vaccination with HA2 protein alone. The HA2-specific immune response obtained after i.p vaccination of mice with CyaA-E5–HA2 comprised antibodies of the IgG1, as well as of IgG2a and IgG2b isotypes, the latter beeing shown to be important mediators of ADCC in mice (Kipps et al., 1985; Denkers et al., 1985). It is, therefore, plausible to speculate that HA2-specific antibodies elicited by the vaccination with CyaA-E5–HA2 might contribute to the protection against IAV also by an indirect mechanism, such as the Ab-dependent cytotoxicity (ADCC) via the Fc region recognition or the FcR-mediated phagocytosis (Huber et al., 2001; Staneková and Varečková, 2010). Inhibition of virus fusion with host cell membranes might be another possible mechanism by which HA2-specific antibodies could neutralize the influenza infection (Varečková et al, 2003b; Gocník et al., 2007; Wang et al., 2010a,b), We showed here that antibodies induced by the immunization with CyaA-E5–HA2 reduced the virus replication. Therefore, we can speculate that they might contribute directly, at least partially, to the milder course of the influenza infection in immunized mice.

It was shown that induction of both $CD4^+$ T cells and $CD8^+$ T cells plays an important role in the recovery from the influenza infection (McMurry et al., 2008). In CyaA-E5–HA2-immunized mice we observed, indeed, an increase in the number of influenza virus-specific IFNγ-secreting splenocytes after restimulation with HA2-specific $CD4^+$ and $CD8^+$ T cell peptides. The induction of HA2-specific $CD8^+$ and $CD4^+$ T cells response was achieved already after the first immunization dose. Two of these epitopes recognized by T cells (the $CD8^+$ epitope $HA2_{93-102}$ and the $CD4^+$ epitope $HA2_{96-104}$) are located in the conserved region 76_{-130} of HA2 that was shown to induce antibody mediated protection against distinct viral subtypes (Wang et al., 2010a,b). Therefore, it can be supposed that the cross-protective potential of this region of HA2 might be enhanced by stimulation of T-cell immune response when CyaA is used as a tool for HA2 antigen delivery. We report here for the first time that a HA2-based vaccine can induce not only a protective humoral response, as shown previously (Gocník et al., 2008; Steel et al., 2010; Wang et al., 2010a,b; Bommakanti et al., 2010), but also induce a $CD8^+$ and $CD4^+$ T cell response that may enhance the protective immunity. These results suggest that the use of adenylate cyclase toxoids as antigen carrier is a valid approach for stimulation of both arms of protective immunity against influenza virus infection.

This conclusion is underpinned by our observation that vaccination with CyaA-E5–HA2 provided earlier clearance of influenza virus from mice lungs and protected mice not only against a lethal infection with homologous, human influenza virus A/Miss H3N2, but also conferred protection against a heterologous and highly pathogenic avian influenza virus A/Chick H7N1. The latter is, indeed, sporadically transmitted from birds to humans and exhibits the potential to start a new influenza pandemic.

It is particularly noteworthy that this report is the first demonstration of the cross-protection mediated by HA2-based vaccine without the need for any adjuvant. It should be stressed in this respect that the T-cell and specific antibody responses were induced by a non-replicating vector, CyaA-E5–HA2, which could previously be reached only after vaccination with live attenuated influenza vaccines, the application of which in humans has some restrictions (in USA high risk groups comprising the old people, newborns as well as pregnant women and immune-deficient patients are excluded) (Stropkovská et al., 2010).

It remains to be explored how readily the here-developed CyaA-E5–HA2-based vaccine can be modified so as to broaden its protective efficacy also against other circulating IAV strains, particularly the H1N1, or the highly pathogenic avian influenza viruses H5N1 and H9N2, respectively. The here selected $CD8^+$ (HA_{93-102}) and $CD4^+$ (HA_{96-104}) T-cell epitopes are highly conserved not only within viruses of group 2 (H3, H4), but conservancy analysis showed also their high homology with viruses of group 1 (H1 – 80% and H5 – 70% vs. H3 subtypes). We assume that after considering the differences in the region $HA2_{23-185}$ derived from HA of any influenza virus within group 1 and upon selection of a suitable combination of the HA2 peptides from each group (e.g. HA2 gp from H1 subtype (group 1) and HA2 gp from H3 subtype (group 2), a protection against influenza viruses from both phylogenetic groups 1 and 2 could be achieved. Such a recombinant CyaA–HA2 based vaccine would represent an alternative to currently available influenza vaccines and might help to minimize the devastating effects of newly emerging highly pathogenic influenza A viruses.

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6.5 PUBLICATION 4

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The adenylate cyclase toxin (CyaA) from *Bordetella pertussis* primarily targets phagocytes expressing CD11b/CD18 (CR3, $\alpha_M\beta_2$ integrin). The toxin delivers its adenylate cyclase (AC) domain into cell cytosol, which has been exploited over the past 20 years for delivery of foreign cargo $CD8^+$ epitopes into the major histocompatibility complex class I (MHC I) presentation pathway. CyaA possesses a unique ability to penetrate the host cell membrane and deliver its AC domain into the cell cytosol directly, without the need for endocytosis. AC domain translocation depends on the plasma membrane potential and on an intact, acylated and calcium-loaded RTX domain. In this work, we examined the role of the AC domain polypeptide in the process of AC penetration into the cell cytosol and in the delivery of heterologous epitopes. We used a set of 18 CyaA constructs with various deletions within the AC domain and an inserted OVA epitope, in order to find out which part of the AC domain was necessary for antigen delivery.

Our results show that the first 371 amino acids are dispensable for the capacity of CyaA to deliver inserted epitopes into DC, implicating that the role of the AC domain during the process of penetration is rather passive. Moreover, the CyaA mutants with heterologous polypeptides in place of the AC domain were able to induce strong antigen specific $CD8^+$ CTL responses *in vivo* in mice and *ex vivo* in human peripheral blood mononuclear cell cultures. These results suggest that the AC domain participates in membrane penetration only passively, while the other part of the CyaA molecule, the RTX domain (residues 374 to 1706) plays a dominant role during the process of CyaA penetration and antigen delivery. This capacity of CyaA to deliver a large heterologous inserts into the cytosol of antigen-presenting cells seems to be quite exceptional, enabling construction of a next generation of CyaA-

derived antigen delivery tools, in which the entire AC domain will be replaced by polyepitope constructs inducing polyvalent $CD8^+$T cell immune responses.

My contribution: Preparation of some recombinant CyaA toxoids, *in vitro* assays testing antigen delivery capacity of toxoids.

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Delivery of Large Heterologous Polypeptides across the Cytoplasmic Membrane of Antigen-Presenting Cells by the *Bordetella* RTX Hemolysin Moiety Lacking the Adenylyl Cyclase Domain

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The *Bordetella* adenylate cyclase toxin-hemolysin (CyaA; also called ACT or AC-Hly) targets CD11b-expressing phagocytes and translocates into their cytosol an adenylyl cyclase (AC) that hijacks cellular signaling by conversion of ATP to cyclic AMP (cAMP). Intriguingly, insertion of large passenger peptides removes the enzymatic activity but not the cell-invasive capacity of the AC domain. This has repeatedly been exploited for delivery of heterologous antigens into the cytosolic pathway of CD11b-expressing dendritic cells by CyaA/AC$^-$ toxoids, thus enabling their processing and presentation on major histocompatibility complex (MHC) class I molecules to cytotoxic CD8$^+$ T lymphocytes (CTLs). We produced a set of toxoids with overlapping deletions within the first 371 residues of CyaA and showed that the structure of the AC enzyme does not contain any sequences indispensable for its translocation across target cell membrane. Moreover, replacement of the AC domain (residues 1 to 371) with heterologous polypeptides of 40, 146, or 203 residues yielded CyaAΔAC constructs that delivered passenger CTL epitopes into antigen-presenting cells (APCs) and induced strong antigen-specific CD8$^+$ CTL responses *in vivo* in mice and *ex vivo* in human peripheral blood mononuclear cell cultures. This shows that the RTX (repeats in toxin) hemolysin moiety, consisting of residues 374 to 1706 of CyaA, harbors all structural information involved in translocation of the N-terminal AC domain across target cell membranes. These results decipher the extraordinary capacity of the AC domain of CyaA to transport large heterologous cargo polypeptides into the cytosol of CD11b$^+$ target cells and pave the way for the construction of CyaAΔAC-based polyvalent immunotherapeutic T cell vaccines.

The 1,706-residue-long adenylate cyclase toxin hemolysin (ACT; also called AC-Hly or CyaA) secreted by the whooping cough agent *Bordetella pertussis* primarily targets the phagocytic myeloid cells expressing the $\alpha_M\beta_2$ integrin receptor CD11b/CD18, such as macrophages, neutrophils, and dendritic cells (16). The toxin directly penetrates the cytoplasmic membrane of cells, without the need for endocytosis (13), and delivers its N-terminal adenylyl cyclase (AC) enzyme domain, which consists of the first 373 residues, to the cytosol (18). Inside cells, the AC binds calmodulin and catalyzes unregulated conversion of cellular ATP to the key signaling molecule cyclic AMP (cAMP), thereby disrupting signaling and bactericidal functions of CD11b$^+$ phagocytes and promoting host colonization by *Bordetella* (50).

The 1,333 carboxy-proximal residues of CyaA constitute an Hly moiety belonging to the RTX (repeats in toxin) family of pore-forming hemolysins and leukotoxins of Gram-negative pathogens (29, 51). Hly accounts for the receptor binding, membrane insertion, and pore-forming activities of CyaA (6, 40). It contains a hydrophobic domain (residues 500 to 700 of CyaA) that forms small cation-selective pores in target cell membranes with a diameter of only 0.6 to 0.8 nm (1, 3, 35, 47). The Hly further harbors two posttranslational palmitoylation sites at lysine residues 860 and 983 (19, 20), where acylation of at least one of them confers on CyaA the capacity to bind its receptor, CD11b/CD18, and penetrate cells (6, 32). Finally, the C-terminal RTX domain of Hly harbors ~40 calcium-binding sites that are formed by glycine- and aspartate-rich nonapeptide repeats. Loading of these sites with Ca^{2+} structures the toxin into the active conformation for target cell interactions (23, 39).

The structure of Hly has not been determined, and the mechanistic details of AC domain penetration across target cytoplasmic membrane remain poorly understood. AC translocation into cells depends on negative plasma membrane potential (36) and does not appear to proceed through the cation-selective pore formed by CyaA (34, 49). It depends, however, on structural integrity of the four predicted transmembrane amphipathic α-helices located between residues 502 to 522 and 565 to 591 of the hydrophobic domain of CyaA (35). These harbor pairs of negatively charged glutamate residues (Glu^{509} plus Glu^{516} and Glu^{570} plus Glu^{581}) that have been found to be directly involved in AC domain translocation across target cell membranes (1, 35).

It has repeatedly been demonstrated that substitution of catalytic residues, or disruption of the ATP-binding site of the AC by dipeptide insertions, does not affect the capacity of the resulting CyaA/AC$^-$ toxoids to translocate the enzymatically inactive AC

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polypeptide across the cell membrane (45). Moreover, the cell-invasive capacity of CyaA was found to be mostly conserved even upon insertion of a broad range of heterologous antigenic polypeptides up to 200 residues in length into defined permissive sites within the AC domain (12, 30, 33). This has been successfully exploited for delivery of numerous AC-inserted passenger antigens into the cytosol of antigen-presenting cells (APCs) for processing by proteasomes and subsequent presentation of the excised major histocompatibility complex (MHC) class I-restricted epitopes on the surface of APCs to cytotoxic $CD8^+$ T lymphocytes (CTL) (9). Immunization with CyaA/AC^- bearing appropriate $CD8^+$ T-cell epitopes, indeed, induced strong and epitope-specific CTL responses, which conferred full prophylactic protection against a lethal lymphocytic choriomeningitis virus challenge (41) or were efficient in tumor immunotherapy in mice (8, 30, 37).

It has previously been shown that the AC domain has to unfold during translocation across the cellular membrane (12). Moreover, the versatility of the AC domain in accommodating and delivering a broad range of heterologous polypeptides into cells suggested that no particular tertiary structure of the AC domain was involved in its cell-invasive capacity. However, it remained unclear whether the AC domain harbors any specific segments playing a mechanistic role in the AC translocation process through interactions with the Hly moiety or the target cell membrane. Here we used a systematic deletion analysis to show that no segment of the AC polypeptide (residues 1 to 371) is essential for its translocation into cells. We found that the AC domain is passively entrained across the cellular membrane by the Hly moiety, which can also translocate large artificial polypeptides, instead of the AC domain, into cells.

MATERIALS AND METHODS

Mice. Male C57BL/6 (Ly 5.2) mice were obtained from a breeding colony at the Institute of Physiology of the ASCR in Prague, Czech Republic. Transgenic OT-I mice and B6.SJL (Ly5.1) mice were bred and kept at the GMO facility of the Institute of Molecular Genetics of the ASCR in Prague. Transgenic OT-I mice were mated with B6.SJL (Ly5.1) mice, and OT-I positive $Ly5.1^+$ littermates were identified. The mice were used at 12 to 20 weeks of age. All animal experiments were approved by the competent Animal Welfare Committee of the Institute of Microbiology of the ASCR, v.v.i., in Prague, Czech Republic. Handling of animals was performed according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National Assembly, Collection of Laws No. 149/2004, inclusive of the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws No. 207/2004, on care and use of experimental animals.

Cell cultures, growth conditions, and handling of cells. DC2.4 and B3Z cell cultures were maintained in RPMI medium containing 10% fetal calf serum (FCS; Gibco) and antibiotic-antimycotic solution (0.1 mg/ml streptomycin, 1,000 units/ml penicillin, and 0.25 μg/ml amphotericin; Sigma-Aldrich). Only adherent cells of the semiadherent B3Z hybridoma were used for the next passage. HLA-A*0201-expressing T2 cells (ATCC CRL-1992) were cultured in standard Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Lonza, Verviers, Belgium), 10% bovine fetal serum, and 1% penicillin-streptomycin (both from Sigma-Aldrich, Prague, Czech Republic).

Peripheral blood mononuclear cells (PBMCs) from 10 cytomegalovirus (CMV)-seropositive donors (anti-CMV IgG antibodies detectable in serum) were used. Healthy donors were positive for human leukocyte antigen A*0201 (HLA-A*0201) and were recruited at the transfusion unit from blood donors that signed an informed-consent form approved by the Ethical Committee of the University Hospital of Brno. The PBMCs were isolated by Histopaque (Sigma-Aldrich, Prague, Czech Republic) gradient centrifugation of anticoagulated blood buffy coats. Viable cells were quantified, cryopreserved in Hanks' balanced salt solution (HBSS) containing 40% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (all from Sigma-Aldrich, Prague, Czech Republic), and stored in liquid nitrogen.

Deletion mutagenesis in the AC domain of CyaA. Deletions in the AC domain were performed according to standard protocols using the plasmid pT7CT7ACT1/E5-mutNdeI. This was derived from pT7CACT1 (33) by placing the ribosome binding site RBS_{10} from gene 10 of bacteriophage T7 upstream of the *cyaA* open reading frame, thus allowing increased production of CyaA protein in *Escherichia coli*. Insertion of a synthetic BamHI linker (5′-GGATCC) between codons 188 and 189 of *cyaA* was used to generate alleles for production of detoxified CyaA/AC^- toxoids, in which the ATP binding site was disrupted by a GlySer dipeptide insert at position 188 of the AC domain as previously described (9). Next, the NdeI restriction site sequence (5′-CATATG), comprising the ATG initiation codon of the *cyaC* gene, was removed by a T-to-A mutation, generating the 5′-CAAATG sequence, to yield the pT7CT7ACT1/E5-mutNdeI plasmid with a unique NdeI restriction site comprising the ATG initiation codon of the *cyaA* gene. Unique BsrGI restriction sites were then introduced at four different permissive sites within the *cyaA* gene, between codons 107 and 108, codons 232 and 233, codons 335 and 336, and codons 423 and 424 (33). Deletions in the 5′ portion of *cyaA* encoding the AC domain were next performed by using combinations of the unique NdeI, BsrGI, BclI, AgeI, and BstBI restriction sites. At the same time, double-stranded synthetic oligonucleotides encoding the $OVA_{257\text{-}264}$ epitope SII NFEKL, corresponding to residues 257 to 264 of ovalbumin and surrounded by unique KpnI and NheI restriction sites, were inserted in frame into the truncated *cyaA* alleles. The generated deletions in the AC domain are summarized in Table 1, where the sequences of the modified portions of CyaA toxoids are given. Further details will be provided upon request.

To extend the flanking sequence of the $OVA_{257\text{-}264}$ epitope (SIINF EKL) in the A18 construct (Table 1), and to introduce a VNGLEQLESII NFEKLTEWTSSNVMEERKIKVYLPR insert, corresponding to residues 249 to 284 of ovalbumin ($OVA_{249\text{-}284}$), a corresponding double-stranded synthetic oligonucleotide was inserted using the KpnI and NheI restriction sites, yielding construct B18 (Table 1).

The control toxoids 108B/AC^-, 233B/AC^-, and 336B/AC^- were prepared by the insertion of the corresponding double-stranded synthetic oligonucleotide, encoding the $OVA_{249\text{-}284}$ peptide, into the permissive BsrGI restriction sites inserted at codons 108, 233, and 336, respectively. The negative control 233B-KP/AC^- was prepared from a doubly mutated CyaA/E570K+E581P/AC^- toxoid variant that was previously obtained in frame from extensive mutagenesis of the CyaA toxin gene (1).

To generate the A+C18 and 233A+C/AC^- constructs (Table 1), the open reading frame encoding a polypeptide composed of conserved segments of the HIV Gag protein (28) was PCR amplified from pGA15 HIV-CON (kind gift of T. Hanke, University of Oxford, Oxford, United Kingdom).

To generate constructs B+D18 and 108A/233D/AC^- (Table 1), the immunodominant epitopes of the human cytomegalovirus (CMV) phosphoprotein 65 (pp65) (46, 52) were assembled into one polyepitope polypeptide (Table 1), and the corresponding codon-optimized synthetic open reading frame was purchased from GenScript (Piscataway, NJ).

The exact sequence of relevant portions of all plasmids used for protein production was systematically verified by DNA sequencing.

Production and purification of CyaA proteins. CyaA toxins and CyaA/AC^- toxoids were produced in *Escherichia coli* BL21/pMM100 (*lac* repressor gene $lacI^q$ carried on pMM100) and purified as described earlier (33). During hydrophobic chromatography, the resin with bound CyaA was repeatedly washed with several bed volumes of 60% isopropanol to remove bacterial endotoxin (48). In the final step, the proteins were eluted with 8 M urea, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0) and stored at −20°C. The endotoxin content of the samples was determined by the *Limulus* amebocyte lysate assay (QCL-1000; Cambrex) according to the

TABLE 1 CyaA/AC$^-$ toxoids

Toxoid[a]	Deletion	Sequence[b]
A1	Δ233–271	SEATGG^{232}**VHGT**SIINFEKL**ASA**^{272}ITDFEL
A2	Δ274–319	MNIGVIT^{273}**GT**SIINFEKL**ASS**^{320}GESQML
A3	Δ321–335	VVSATG^{320}**GT**SIINFEKL**ASGVH**^{336}QQRGEG
A4	Δ108–271	SSLAHG^{107}**VHGT**SIINFEKL**ASA**^{272}ITDFEL
A5	Δ108–319	SSLAHG^{107}**VHGT**SIINFEKL**ASS**^{320}GESQML
A6	Δ233–319	SEATGG^{232}**VHGT**SIINFEKL**ASS**^{320}GESQML
A7	Δ274–335	MNIGVIT^{273}**GT**SIINFEKL**ASGVH**^{336}QQRGEG
A8	Δ336–371	LKEYIG^{335}**VRGT**SIINFEKL**AS**^{372}RSKFSP
A9	Δ321–371	VVSATG^{320}**GT**SIINFEKL**AS**^{372}RSKFSP
A10	Δ274–371	MNIGVIT^{273}**GT**SIINFEKL**AS**^{372}RSKFSP
A11	Δ233–371	SEATGG^{232}**VHGT**SIINFEKL**AS**^{372}RSKFSP
A12	Δ108–371	SSLAHG^{107}**VHGT**SIINFEKL**AS**^{372}RSKFSP
A13	Δ2–107	M^1**GT**SIINFEKL**ASGVH**^{108}HTAVDL
A14	Δ2–232	M^1**GT**SIINFEKL**ASGVH**^{233}LDRERI
A15	Δ2–271	M^1**GT**SIINFEKL**ASA**^{272}ITDFEL
A16	Δ2–319	M^1**GT**SIINFEKL**ASS**^{320}GESQML
A17	Δ2–335	M^1**GT**SIINFEKL**ASGVH**^{336}QQRGEG
A18	Δ2–371	M^1**GT**SIINFEKL**AS**^{372}RSKFSP
B18	Δ2–371	M^1**GTVNGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRAS**^{372}RSKFSP
B19	Δ2–424	M^1**VNGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRMYT**^{425}AVEAAE
A+C18	Δ2–371	*M^{1}EEKAFSPEVIPMFTALSEGATPQDLNTMLNTVGGHQAAMQMLKDTINEEAAEWDRIYKRWIILGLNKIV RMYSPVSILDIRQGPKEPFRDYVDRFARN***SG**SIINFEKL**RN***CRAPRKKGCWKCGKEGHQMKDCTERQA NFLGKIWPS*^{372}RSKFSP
B+D18	Δ2–371	M^1**GTVNGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRASRRTS***LNIPSINVHHYPSFVFPTKDVALRHVIG DQYVKVYLESFCEDVPSGKLFMKPGKISHIMLDVAFTSHEHYSEHPTFTSQYRIQGKLEYVTTERKTPR VTGGGAMAGASTSATWPPWQAGILARNLVPMVATVQGQNLKYQEFFWDANDIYRIFAE***RRAS**^{372}RSKFSP
108A/233D/AC$^-$		SSLAHG^{107}**VLSIINFEKLVH**^{108}HTAVDL.....SEATGG^{232}**VQRRTS***LNIPSINVHHYPSFVFPTKDVALRHVI GDQYVKVYLESFCEDVPSGKLFMKPGKISHIMLDVAFTSHEHYSEHPTFTSQYRIQGKLEYVTTERKTPRVT GGGAMAGASTSATWPPWQAGILARNLVPMVATVQGQNLKYQEFFWDANDIYRIFAE***RRDVH**^{233}LDRERI
108B/AC$^-$		SSLAHG^{107}**VQLTGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRIVH**^{108}HTAVDL
233A+C/AC$^-$		SEATGG^{232}*MEEKAFSPEVIPMFTALSEGATPQDLNTMLNTVGGHQAAMQMLKDTINEEAAEWDRIYKRWIIL GLNKIVRMYSPVSILDIRQGPKEPFRDYVDRFARN***SG**SIINFEKL**RN***CRAPRKKGCWKCGKEGHQMKDCTE RQANFLGKIWPS*^{233}LDRERI
233B/AC$^-$ and 233B–KP/AC$^-$		SEATGG^{232}**VQLTGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRIVH**^{233}LDRERI
336B/AC$^-$		LKEYIG^{335}**VQLTGLEQLE**SIINFEKL**TEWTSSNVMEERKIKVYLPRIVH**^{336}QQRGEG

[a] The constructs were enzymatically inactive either due to a GlySer dipeptide insert between residues 188 and 189 of the AC domain (AC$^-$) or due to deletions abolishing the AC enzyme activity.

[b] Each entry includes the insertion point, insert sequence, and flanking sequence. The sequences of CD8$^+$ epitopes of OVA (SIINFEKL) and CMV (NLVPMVATV) are underlined, and the inserted flanking residues are in bold. In the A+C18 and 233A+C/AC$^-$ constructs, the 135-residue artificial sequence assembled from the conserved portions of HIV Gag protein, also comprising T cell epitopes (not used), is in italics. In the B+D18 and 108A/233D/AC$^-$ constructs, the 155-residue sequence composed of conserved CD4$^+$ and CD8$^+$ epitopes of the CMV pp65 phosphoprotein is in italics. The numbers indicate the corresponding residue positions in the intact CyaA polypeptide sequence.

manufacturer's instructions and was below 100 endotoxin units (EU)/mg of purified protein.

On-column biotinylation of CyaA/AC$^-$ toxoids was performed after the DEAE-Sepharose purification step as previously described (49).

Cell binding and cAMP elevation activity of enzymatically active CyaA toxins. For determination of cell binding of enzymatically active CyaA toxins, DC2.4 cells (2×10^6/ml) were incubated in the presence of 30 nM CyaA in Dulbecco's modified Eagle's medium (DMEM) without FCS for 30 min on ice. Cells were washed to remove unbound toxin, and CyaA binding was assessed by determining cell-associated adenylate cyclase enzyme activity in the presence of 1 μM calmodulin as described previously (26). The cAMP-elevating capacity of CyaA was assessed on DC2.4 cells (3×10^5 per well) incubated with various concentrations of CyaA in DMEM without FCS at 37°C for 30 min. The reaction was stopped by the addition of 0.2% Tween 20 in 50 mM HCl, and the intracellular cAMP concentration was determined by an antibody competition immunoassay, as described elsewhere (2).

Cell binding of enzymatically inactive CyaA/AC$^-$ toxoids. DC2.4 cells (5×10^5 per well) in 100 μl of DMEM with 1% FCS were incubated with biotinylated CyaA/AC$^-$ toxoids for 30 min on ice. Unbound toxoid was removed by repeated washing, and cells were stained with phycoerythrin (PE)-conjugated streptavidin (Exbio) at an 1:400 dilution (50 μl/well) for 30 min on ice. CyaA/AC$^-$ binding was analyzed by flow cytometry using a FACS LSR II instrument (BD Biosciences) and FlowJo version 7.2.1 (Treestar, Inc.). To block CyaA/AC$^-$ binding to the CD11b/CD18 receptor, DC2.4 cells were preincubated for 30 min on ice with 10 μg/ml of the anti-mouse CD11b-specific monoclonal antibody M1/70 (eBioscience) prior to addition of CyaA (16).

Presentation of OVA$_{257\text{-}264}$ epitope to the B3Z hybridoma *in vitro*. For *in vitro* presentation of antigens, the immortalized dendritic cell line DC2.4, generated from bone marrow cells of C57BL/6 mice, was used (44). OVA$_{257\text{-}264}$ presentation was determined as β-galactosidase production in B3Z hybridoma CD8$^+$ T cells, which carry a *lacZ* reporter gene under the control of the interleukin 2 (IL-2) promoter NF-AT elements, activated upon TCR recognition of the OVA$_{257\text{-}264}$ peptide (SIINFEKL) on the murine H-2K^b MHC class I molecules (25). DC2.4 cells (10^5/well) were seeded in DMEM with 10% FCS in 96-well plates and pulsed for 4 h at 37°C with various concentrations of toxoids, OVA peptide (purity ≥ 95%; Sigma-Genosys), or ovalbumin protein (albumin from chicken egg white; Sigma-Aldrich). Alternatively, toxoids were preincubated at the

indicated concentration in 200 μl of DMEM containing 10% FCS with 5 μg/ml of 3D1 or the control isotype TU-01 monoclonal antibody (MAb) (Exbio, Prague, Czech Republic) at room temperature for 15 min, before 150 μl of the medium with toxoids and antibody was used to cover 10^5 adherent DC2.4 cells in a 96-well plate for 4 h. The cells were then washed with phosphate-buffered saline (PBS) and further cultured with B3Z hybridoma (10^5 cells/well) in RPMI medium. After 18 h of incubation at 37°C in a humidified 5% CO_2 atmosphere, cultures were lysed by addition of 100 μl per well of PBS containing 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% Nonidet P-40, and 0.15 mM chlorophenol red-β-D-galactopyranoside (CPRG; Sigma-Aldrich), and the β-galactosidase activity was determined as described previously (24). Absorbance was read at 570 nm.

Measuring of intracellular concentration of Ca^{2+} ions. Calcium influx into cells was measured as previously described (10).

Isolation of detergent-resistant membrane and Western blotting. Detergent-resistant membranes (coalesced lipid rafts) were separated by flotation in discontinuous sucrose density gradients, and toxoids were detected in Western blots using the 9D4 antibody as described previously (4).

$CD8^+$ T cell activation *in vivo*. Purified $CD8^+$ T cells from OT-I $Ly5.1^+$ mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and injected intravenously (i.v.) into C57BL/6 (Ly5.2) mice at 1.1×10^6 to 1.5×10^6 cells per mouse. Next day the mice were injected intraperitoneally (i.p.) with PBS and 2 nmol of $OVA_{257\text{-}264}$ peptide (SIINFEKL; MBL International, Woburn, MA) plus poly(I·C) (75 μg) and i.v. with tested purified toxoids (0.3 nmol). Each dose of tested purified toxoids in 8 M urea, 2 mM EDTA, and 50 mM Tris-HCl (pH 8.0) was placed into a separate tube, kept on ice in a small volume (25 to 30 μl), and diluted up to 300 μl with ice-cold PBS immediately before administration. Mice were euthanized 4 days after injection of tested samples, and their spleen cells were harvested.

To determine the expansion of adoptively transferred $CD8^+$ $Ly5.1^+$ cells, spleen cells were resuspended in FACS buffer (PBS with 2% FCS, 2 mmol EDTA, and 0.05% sodium azide), blocked with 10% mouse serum for 30 min on ice, and stained with fluorochrome-labeled MAbs CD8-A700, CD8-PerCP (peridinin chlorophyll protein), CD45.1-allophycocyanin, and CD25-PE (eBioscience, San Diego, CA) for 30 min on ice in the dark. Cells were then washed twice in FACS buffer and transferred into buffer with 4% paraformaldehyde.

For determination of functionality of expanded $CD8^+$ T cells, spleen cells were restimulated *in vitro* with 1 μM SIINFEKL peptide for 2 h, followed by the addition of brefeldin A (1 μg/ml) to block the extracellular release of gamma interferon (IFN-γ) for 4 h. Cells were stained as described above, fixed, and permeabilized in fixation and permeabilization buffer (eBioscience, San Diego, CA), blocked with 2% mouse serum for 10 min on ice, and incubated with IFN-γ–PE and granzyme B–PE (eBioscience) for 30 min on ice in the dark. Flow cytometry was performed using an LSRII instrument (BD Biosciences, Mountain View, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Activation of CMV-specific $CD8^+$ T cells. To monitor CMV-specific T cell activation, PBMCs from CMV-seropositive donors were incubated with various toxoids (5 nM) in high-glucose DMEM with L-glutamine (Lonza, Verviers, Belgium), 8% male human AB^+ serum, and 1% penicillin-streptomycin (both from Sigma-Aldrich, Prague, Czech Republic) at a concentration of 2×10^6 per ml in a 10% CO_2 atmosphere at 37°C for 6 h. After 3 h, 1 μl/ml of BD GolgiPlug (containing brefeldin A; BD Biosciences, San Jose, CA) was added. PBMCs were stained with the fluorochrome-labeled MAbs CD3-PeCy7 (Immunotech, Marseille, France) and CD8-allophycocyanin (Exbio, Prague, Czech Republic) for 20 min on ice. Cells were washed and fixed with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Cells were stained intracellularly with anti-IFN-γ–PE MAb (BD Biosciences) for 30 min on ice. The percentage of $CD8^+$ IFN-γ^+ T cells was determined by flow cytometry using FACS Canto II (BD Biosciences). Data were processed using BD FACS DIVA software, version 5.0.1 (2006) (BD Biosciences). In parallel, PBMCs were also incubated with toxoids in the absence of BD GolgiPlug for 18 h, and the concentration of IFN-γ released into in culture medium was determined by cytometric bead array (CBA) using a human IFN-γ Flex set (BD Biosciences) according to the manufacturer's instructions. Data were analyzed with FCAP array software (2007) and BD FACS array software (2007) (BD Biosciences).

Clonal expansion of CMV-specific $CD8^+$ T cells was assessed after 7 days of PBMC stimulation with 5 nM toxoids by staining with allophycocyanin-labeled HLA-A*0201 NLVPMVATV-loaded MHC I pentamers according to the manufacturer's instructions (Proimmune, Oxford, United Kingdom). PBMCs were further stained with anti-CD3–PeCy7 (Immunotech, Marseille, France) and anti-CD8–PE (Exbio, Prague, Czech Republic) monoclonal antibodies for 20 min on ice. The percentage of pentamer-specific $CD8^+$ T cells was determined by flow cytometry using FACS Canto II and FACS DIVA software, version 5.0.1 (2006).

Functional characterization of clonally expanded CMV-specific $CD8^+$ T cells. PBMC suspensions from CMV-seropositive donors were subjected to antigenic stimulation with various toxoids (5 nM) for 7 days. T2 target cells were loaded with 10 μg/ml of CMV peptide (HLA*A0201 restricted; NLVPMVATV) or HIV-1 reverse transcriptase (Pol) peptide (ILKEPVHGV) for 2 h, washed, and used for restimulation of PBMCs at an effector-to-target ratio of 10:1 for 4.5 h. After 1.5 h, 1 μl/ml of BD GolgiPlug (containing brefeldin A; BD Biosciences) was added. Cells were stained with anti-CD3–PeCy7 (Immunotech, Marseille, France) and anti-CD8–allophycocyanin (Exbio, Prague, Czech Republic) MAbs for 25 min on ice, and $CD3^+$ T cells were sorted using a FACS ARIA instrument (BD Biosciences). The sorted cells were fixed with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions and stained with anti-IFN-γ–PE MAb (BD Biosciences) for 30 min at 4°C. The percentage of $CD8^+$ IFN-γ^+ T cells was determined by flow cytometry using FACS Canto II and BD FACS DIVA software. To determine percentage of CMV-specific $CD8^+$ IFN-γ^+ T cells, the percentage of $CD8^+$ IFN-γ^+ T cells restimulated by T2 target cells loaded with an irrelevant HIV-1 Pol peptide (ILKEPVHGV) was subtracted from the percentage of $CD8^+$ IFN-γ^+ T cells restimulated by T2 target cells loaded with the CMV peptide (NLVPMVATV). In parallel, effector and target cells were cultivated without addition of BD GolgiPlug for 9 h, and the concentration of IFN-γ released into culture supernatants was measured using a cytometric bead array, as described above. The background production of IFN-γ resulting from restimulation with T2 target cells loaded with irrelevant HIV-1 Pol peptide was subtracted.

For determination of cytotoxic activity of the clonally expanded $CD8^+$ T cells, T2 cells were labeled with 1 μM CFSE in PBS with 0.1% bovine serum albumin (BSA) for 10 min at 37°C. Labeling was stopped by addition of male human AB^+ serum (Sigma-Aldrich, Prague, Czech Republic), and cells were washed twice and resuspended in DMEM with 2% male human AB^+ serum. CFSE-labeled T2 cells were loaded with 10 μg/ml of CMV peptide (HLA*A0201 restricted; NLVPMVATV), or the control HIV-1 Pol peptide (ILKEPVHGV) for 2 h and washed prior to use. Antigenic stimulation of PBMCs with CyaA toxoids (5 nM) was performed for 7 days, and cells were washed and coincubated with 4×10^4 of CFSE-labeled and peptide-loaded T2 targets at effector-to-target ratios of 1:1, 5:1, and 10:1. After 3 h of coincubation, cells were stained with 1 μg/ml of propidium iodide (PI) (Sigma-Aldrich, Prague, Czech Republic), and the percentage of dead PI^+ $CFSE^+$ cells was determined by flow cytometry using FACS Canto II and BD FACS DIVA software. Specific cytotoxicity was determined as the percentage of dead target cells loaded with the CMV peptide minus the percentage of dead target cells loaded with HIV peptide.

Statistical analysis. The data were analyzed with STATISTICA 8 software (StaSoft, Prague, Czech Republic). The Mann-Whitney test was used for comparison of two groups, and the Kruskal-Wallis test was used for comparison of three or more groups. Experimental data were compared

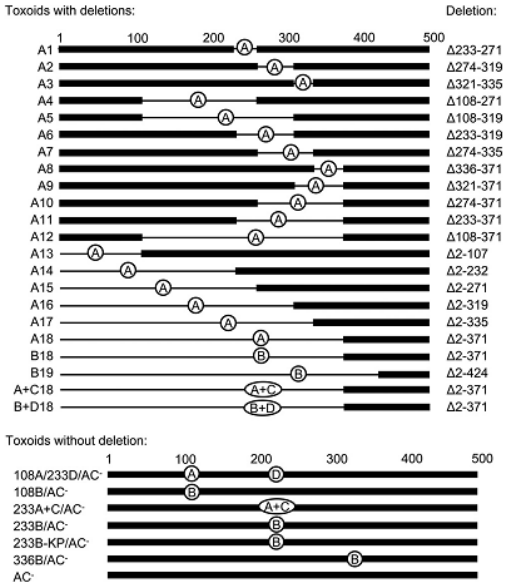

FIG 1 Schematic depiction of toxoid constructs. Deletions in toxoids in which the removed portions were replaced by the inserted antigens are indicated by thin lines (only the first 500 residues of CyaA are depicted). The individual inserts are labeled A to D, corresponding to the names of the constructs. Construct sequences are summarized in Table 1, where exact sequences of all cargo inserts are given in full. Briefly, A stands for the $CD8^+$ CTL epitope $OVA_{257-264}$ (SIINFEKL), used as a marker, B stands for the $OVA_{249-284}$ (SIINFEKL with extended flanking sequence), C stands for the artificial cargo polypeptide of 135 residues assembled from conserved segments of the HIV Gag protein (28), and D stands for a 155-residue artificial polypeptide composed of $CD8^+$ and $CD4^+$ T cell epitopes from the pp65 protein of CMV.

to those obtained with the corresponding controls, and a *P* value of 0.05 or less was considered statistically significant.

RESULTS

All information for translocation of the AC domain is contained within the Hly moiety of CyaA. To map the segments of the AC domain that might be involved in its translocation across the cytoplasmic membrane of $CD11b^+$ target cells, we generated a comprehensive set of 18 overlapping deletions within the AC domain of CyaA. As schematically depicted in Fig. 1 and summarized in Table 1, this construct series, designated A1 to A18, comprised toxoids with limited internal deletions of up to 46 residues (A1, A2, and A3), as well as constructs with rather extensive internal deletions of up to 212 residues (A4, A5, A6, and A7). Moreover, deletions systematically extending from either the carboxy-terminal (A8 to A12) or the amino-terminal (A13 to A18) extremity of the 371-residue AC domain of CyaA were engineered.

Since these deletions eliminated the AC enzyme activity of CyaA, the capacity to elevate cAMP concentration in cells could not be used for assessment of the cell-invasive capacity of such constructs, and a surrogate assay had to be used. To this end, the A1 to A18 toxoids were tagged by the $OVA_{257-264}$ epitope (SIINF EKL) from ovalbumin (Table 1), inserted in place of the deleted AC domain portions. It has previously been established that CyaA delivers its AC domain directly across the cytoplasmic membrane into the cytosol of $CD11b^+$ antigen-presenting cells, such as dendritic cells, where the AC is processed by the cytosolic proteasome. The excised $OVA_{257-264}$ epitope can then enter the classical pathway for MHC class I-restricted antigenic presentation (17), which can be measured as the extent of stimulation of the B3Z $CD8^+$ T hybridoma cells recognizing H-$2K^b$ molecules loaded with the $OVA_{257-264}$ epitope (17, 33, 42). Therefore, the capacity of $CD11b^+$ dendritic cells to stimulate B3Z cells *in vitro* can be used as a measure of the capacity of CyaA to deliver the OVA-tagged AC polypeptide into cytosol of APCs.

To verify that this assay can be specifically used for determination of translocation of the OVA-tagged AC segments into cytosol of APCs, we compared the B3Z-stimulatory capacity of DC2.4 cells incubated with an intact cell-invasive 233B/AC^- toxoid or with a doubly mutated 233B/E570K+E581P/AC^- (233B-KP/AC^-) toxoid variant. The latter harbored a debilitating E570K+E581P combination of residue substitutions in the pore-forming domain, which was previously shown to preserve full receptor-binding capacity of the toxin while abolishing its capacity to deliver the AC domain into cytosol of cells and form pores in cellular membrane (4, 49). As documented in Fig. 2A, the enzymatically active (AC^+) CyaA/E570K+E581P (CyaA-KP) construct exhibited the full capacity to bind to DC2.4 cells through CD11b/CD18, and this interaction was blocked by the CD11b-specific antibody M1/70 (17). In contrast to intact CyaA, however, CyaA-KP exhibited only a residual capacity (<2%) to translocate the AC domain into the cytosol of DC2.4 cells and elevate cytosolic cAMP (Fig. 2B) (4). As shown in Fig. S1 in the supplemental material, the enzymatically inactive AC^- toxoid constructs 233B/AC^- and 233B-KP/AC^-, bearing a GlySer dipeptide insert between residues 188 and 189 to disrupt the ATP-binding site (9), also exhibited an identical capacity to bind DC2.4 cells in an M1/70-inhibitable manner. As shown in Fig. 3A, 233B-KP/AC^- failed to deliver the $OVA_{257-264}$ epitope into DC2.4 cells and failed to promote stimulation of B3Z cells, in contrast to the three cell-invasive 108B/AC^-, 233B/AC^-, and 336B/AC^- toxoids bearing the OVA epitope inserted at residues 108, 233, and 336 of the AC domain, respectively (33). These control toxoids promoted a readily detectable B3Z response at a <0.1 nM concentration, while no B3Z stimulation was observed even at 100-fold-higher concentrations of 233B-KP/AC^-. As further shown in Fig. 3B, a molar concentration of free soluble ovalbumin protein (0.1 mM) over five orders of magnitude higher had to be used to induce a detectable B3Z hybridoma response. Therefore, cross presentation of $OVA_{257-264}$ due to macropinocytic uptake or receptor-mediated antigen endocytosis was undetectable under the conditions used and did not interfere with the assay. It can hence be concluded that mere toxoid binding to the CD11b/CD18-expressing APCs did not result in presentation of the $OVA_{257-264}$ epitope and that stimulation of a B3Z T cell response strictly depended on the specific capacity of the used toxoids to translocate the epitope across the cellular membrane into the cytosol of DC2.4 cells.

Once the robustness and specificity of the used OVA/AC translocation assay had been demonstrated, the capacity of the A1 to A18 deletion constructs to deliver the $OVA_{257-264}$ epitope into the cytosol of DC2.4 cells could be assessed. As shown in Fig. 4A and Fig. S2 in the supplemental material, all but one of the 18 tested

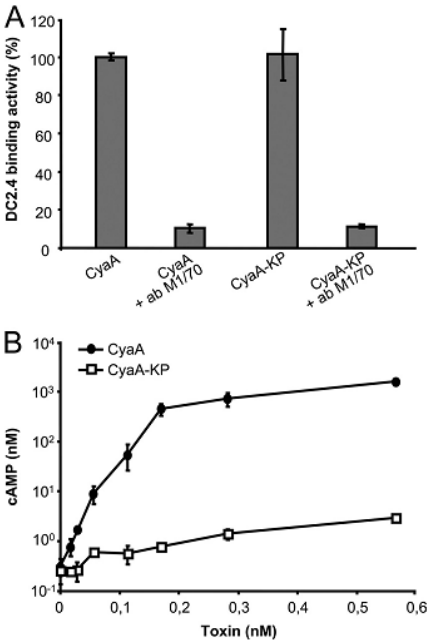

FIG 2 Combination of the E570K and E581P substitutions selectively abolishes the AC domain translocation capacity of CyaA without affecting toxin binding to CD11b/CD18-expressing cells. (A) Mouse CD11b$^+$ DC2.4 cells (2×10^6/ml) were incubated in the presence of 30 nM CyaA (AC$^+$) in DMEM without FCS for 30 min on ice. After removal of the unbound toxin, the cell-associated adenylate cyclase enzyme activity was determined. To block the CD11b/CD18 receptor, cells were preincubated for 30 min on ice with 10 μg/ml of the CD11b-specific antibody M1/70 prior to addition of CyaA. DC2.4 binding activities are expressed as percentages of wild-type CyaA binding activity and are means ± standard deviations from two independent determinations performed in duplicate ($n = 4$). (B) cAMP levels in DC2.4 cells were determined upon 30 min of incubation of DC2.4 cells (3×10^5 per well) with the indicated toxin concentrations. Values are means ± standard deviations from two independent determinations performed in triplicate ($n = 6$).

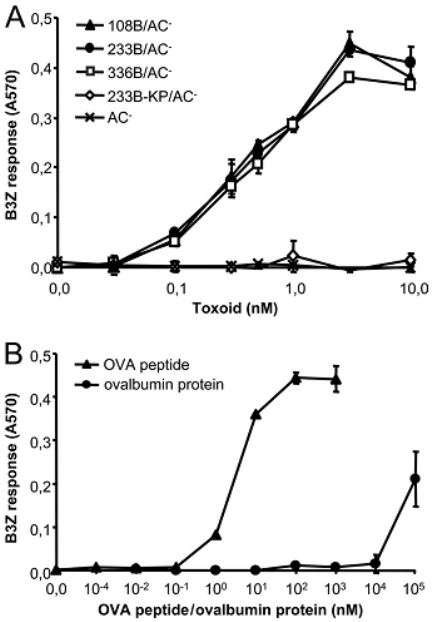

FIG 3 The capacity to translocate the AC domain into the cytosol is required for efficient delivery of the $OVA_{257-264}$ epitope for presentation on MHC class I molecules. DC2.4 cells were incubated with the indicated concentrations of toxoids (A), the $OVA_{257-264}$ peptide, or free soluble ovalbumin (B) for 4 h. After being washed with PBS, the DC2.4 cells were further cultured for 18 h with the B3Z CD8$^+$ T-hybridoma cells selectively recognizing cell surface-presented complexes of the H-2K^b MHC class I molecules with bound $OVA_{257-264}$ peptide. Stimulation of B3Z cells was assayed as the amount of accumulated β-galactosidase. The means ± standard errors (SE) of duplicate samples, representative of three independent determinations, are shown.

constructs (A1 to A17) reproducibly exhibited a comparable capacity to deliver the $OVA_{257-264}$ epitope for presentation on MHC class I molecules, as the 233B/AC$^-$ control toxoid, which was unaffected by the size and location of the various deletions within the AC domain. The only construct with impaired capacity to deliver the epitope was A18 protein, in which the residues 1 to 371 were replaced by the $OVA_{257-264}$ epitope peptide. As shown in Fig. 4A and Fig. S2 in the supplemental material, ~10-fold more toxoid A18 (10 nM) than A1 to A17 was needed for induction of a B3Z response.

Interestingly, while the A17 construct had the first 335 residues of CyaA deleted, A18 carried a larger deletion, comprising 371 of the 373 N-terminal residues that form the minimal functional structure of the AC enzyme (18). The segment between residues 336 and 371 of the AC was, however, unlikely to be specifically required for AC domain translocation and $OVA_{257-264}$ delivery into DC2.4 cytosol. The same segment was missing in several constructs that exhibited a full capacity to deliver the OVA epitope into DC2.4 cells, such as A8 (Fig. 4A) and A9 to A12 (see Fig. S2 in the supplemental material). Hence, it appeared more likely that when delivered by the A18 construct, the $OVA_{257-264}$ epitope remained buried in the plasma membrane and was inefficiently excised by intracellular proteases.

To test this hypothesis, a 36-residue $OVA_{249-284}$ polypeptide (VNGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPR) comprising the natural flanking regions from ovalbumin was used to derive from the A18 construct the B18 protein, in which the number of residues separating the $OVA_{257-264}$ epitope from the fusion point at position 372 of the CyaA moiety was extended from 2 to 22 residues. As shown in Fig. 4B, this extension restored the antigen delivery capacity of the truncated CyaA moiety, as the B18 toxoid exhibited a capacity to deliver $OVA_{257-264}$ at a level comparable to that of the 233B/AC$^-$ control. As further shown in Fig. 4B, however, when the N-terminal deletion in CyaA was extended up to residue 424 in a B19 construct, still exhibiting a nearly full (~60%) capacity to bind cells through interaction with CD11b/CD18 (see Fig. S1 in the supplemental material), the B19 protein was unable to deliver the $OVA_{257-264}$ epitope for presentation to B3Z T hybridoma cells by APCs.

To further verify that the $OVA_{257-264}$ epitope delivered by B18

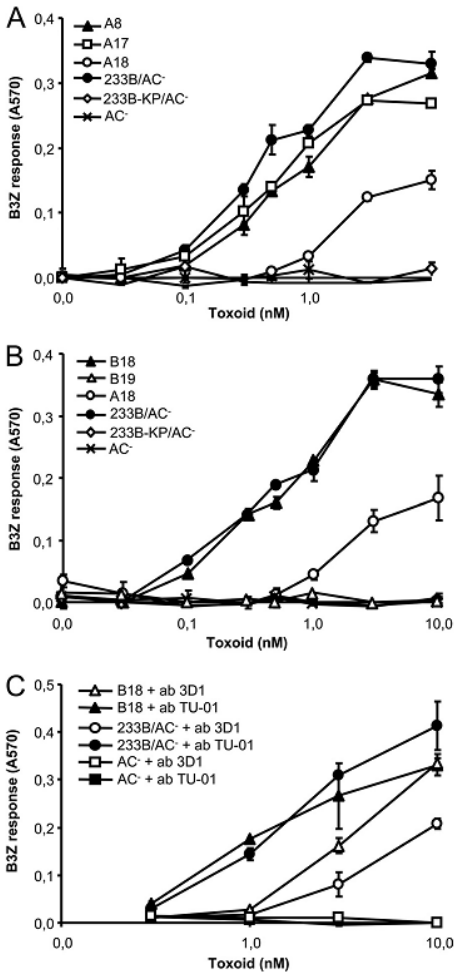

FIG 4 The entire AC domain polypeptide sequence is dispensable for CyaA-mediated delivery of the $OVA_{257-264}$ epitope for MHC class I-restricted antigenic presentation. DC2.4 cells were incubated with the indicated concentrations of the toxoids, and their capacity to deliver the $OVA_{257-264}$ for antigenic presentation was assessed as described above. (C) Toxoids were preincubated with 5 μg/ml of 3D1 or the control isotype TU-01 antibody at room temperature for 15 min before addition to DC2.4 cells. The means ± SE of duplicate samples, representative of three independent determinations, are shown.

reached the cytosol of APC due to translocation across the cellular membrane, the antigen delivery capacity of the B18 protein complexed with the 3D1 monoclonal antibody was determined. 3D1 was previously shown to block translocation of the AC domain across the cell membrane because of specific binding to the segment delimited by residues 373 to 400, which links the AC domain to the Hly moiety of CyaA (27). Moreover, we found recently that 3D1 binding locks CyaA in a translocation-intermediate conformation, in which the AC domain remains outside the cytosol and is inaccessible to processing by cytosolic proteases, while still being able to conduct extracellular Ca^{2+} ions into cells (4). As shown in Fig. 4C, by comparison to a control isotype antibody TU-01, preincubation with the 3D1 MAb reproducibly inhibited the capacity of the B18 and 233B/AC^- toxoids to deliver the $OVA_{257-264}$ epitope for presentation by DC2.4 cells to B3Z $CD8^+$ T cells. It can hence be concluded that extension of the N-terminal sequence of the A18 construct by an artificial 20-residue peptide spacer conferred on the B18 construct the capacity to deliver the $OVA_{257-264}$ epitope across the cytoplasmic membrane of APCs and rendered the cargo SIINFEKL peptide accessible for excision and presentation on APCs.

Collectively, the above-discussed results show that of the 373 residues in the AC polypeptide, the first 371 residues do not contain any segments specifically involved in AC domain translocation across cell membrane. All structural information required in this process appears to be confined within the residues 372 to 1706 of CyaA, which consists of the linker segment between residues 374 to 400 and the 1,306 C-terminal residues of the Hly moiety of CyaA. Indirectly, these data also suggest that the linker segment between residues 373 to 400 is inserted into the cell membrane or may even translocate across it.

CyaAΔAC, which has the AC domain replaced by an unrelated polypeptide, conducts Ca^{2+} ions into cells and relocates into the lipid rafts. Recently we showed that upon interaction with its receptor CD11b/CD18, the CyaA toxin is inserted into macrophage membranes as a translocation intermediate that forms a novel path conducting extracellular Ca^{2+} ions across the cytoplasmic membrane (10). The incoming Ca^{2+} was then shown to activate the cytosolic protease calpain, which by processing the talin tether liberates the toxin-receptor complex for relocation into lipid rafts, from which the translocation of the AC polypeptide across membrane is completed (4). It was therefore important to examine whether the truncated B18 protein, lacking 371 of the 373 residues of the AC enzyme polypeptide, was still able to conduct Ca^{2+} ions into cells and relocate into lipid rafts. As shown by comparison to 233B/AC^- in Fig. 5A, the truncated B18 toxoid exhibited a severalfold-lower but clearly detectable capacity to conduct extracellular Ca^{2+} ions into cells. In contrast, the B19 construct, which was unable to deliver the epitope for presentation, was as unable to promote Ca^{2+} influx as the construct 233B-KP/AC^-, which is defective in formation of the translocation precursor and delivery of the AC domain into cytosol. These results are consistent with our previous report showing that segments of the AC domain inserted into and translocating across the cell membrane participate in formation of the conduit enabling influx of extracellular Ca^{2+} ions (10). Moreover, the structural and conformational alterations of the AC domain due to peptide inserts were previously shown to also affect the calcium-conducting properties of CyaA (10). This is also corroborated here by the observation of different amplitudes and time courses of the Ca^{2+} influx curves promoted by AC^-, 233B/AC^-, and B18 constructs.

Consistent with its capacity to promote Ca^{2+} influx, the B18 toxoid still relocated into lipid rafts in cell membranes and floated in sucrose density gradient separations with the detergent-resistant membrane fraction (coalesced lipid rafts), like the intact 233B/AC^- and the lipid raft marker NTAL (Fig. 5B). In contrast, the 233B-KP/AC^- mutant remained in the bulk membrane phase together with the marker CD71 (transferrin receptor). However,

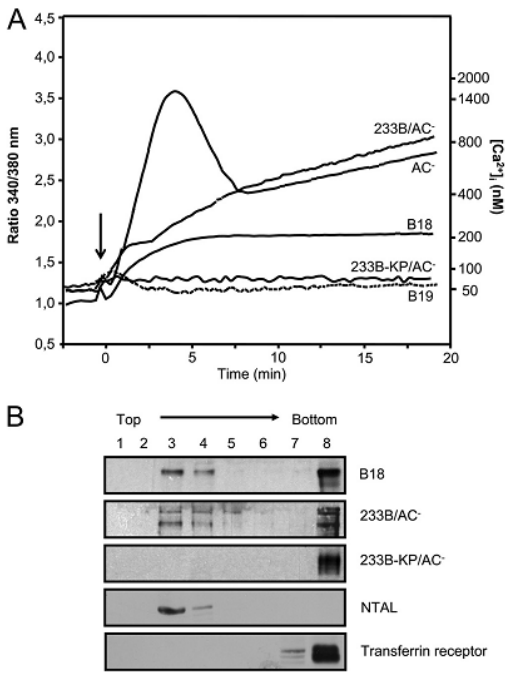

FIG 5 The CyaAΔAC toxoid delivering $OVA_{257-264}$ epitope conducts Ca^{2+} ions into cells and associates with lipid rafts. (A) J774A.1 cells were loaded with the calcium probe Fura-2/AM at a 3 μM final concentration at 25°C for 30 min. After cells were washed in HBSS medium, toxoid variants (17 nM) were added at time zero (arrow), and the time course of calcium entry into the cytosol (elevation of intracellular $[Ca^{2+}]$) was monitored by spectrofluorimetry (ratiometric measurement) as described elsewhere (10). (B) J774A.1 cells were incubated for 10 min with the indicated toxoids (1 nM). Detergent-resistant membrane microdomains were extracted with cold Triton X-100 and separated by flotation in a sucrose density gradient. The toxoids were detected in gradient fractions with the 9D4 antibody using Western blots. Results of one representative experiment of two are shown.

the processing of the B18 protein present in the raft fraction could not be assessed due to poor resolution of the full-length ~150-kDa (1,375-residue) B18 protein and its processed form in the 7.5% acrylamide gels used for Western blot analysis.

CyaAΔAC toxoids with the AC domain replaced by a CTL polyepitope sequence prime specific mouse and human CTL responses. It was next important to examine whether the CyaAΔAC toxoids, having the first 371 residues of the AC domain replaced by large artificial polypeptides, were still able to deliver the cargo CTL epitopes for presentation to specific $CD8^+$ T cells. Therefore, an A+C18 construct was generated, in which the $OVA_{257-264}$ epitope was integrated into an artificial 135-residue antigen sequence derived from the HIV Gag protein (28). The capacity of the A+C18 construct (for the sequence, see Table 1) to deliver the $OVA_{257-264}$ marker epitope for antigenic presentation was next assessed *in vivo* as the capacity of the A+C18 protein to expand naïve OT-I $CD8^+$ T cells expressing the T cell receptor specifically recognizing the $OVA_{257-264}$ epitope in complex with H-$2K^b$ MHC class I molecules. To this end, 1.5×10^6 purified CFSE-labeled $CD8^+$ T cells from OT-I (Ly5.1) transgenic mice were injected i.v. into syngeneic C57BL/6 (Ly5.2) mice, and 24 h after the transfer, the mice were immunized by i.v. injection of selected CyaA toxoids. Presentation of toxoid-delivered epitope to OT-I cells was then measured as specific stimulation of OT-I cells. As shown by comparison to PBS-injected mice (Fig. 6A), 4 days after administration of 0.3 nmol of full-length control toxoids (233B/AC^- and 233A+C/AC^-) or of the toxoids lacking the AC domain (B18 and A+C18), a very strong, 100- to 200-fold *in vivo* expansion (mean CFSE label intensity decrease per cell) of the adoptively transferred $Ly5.1^+$ $CD8^+$ T cells was observed. Moreover, the expanded OT-I $CD8^+$ T cells were functional in terms of IFN-γ and granzyme B production after restimulation (Fig. 6B).

The expanded $CFSE^{low}$ cells represented 2.5% to 4.5% of all cells in the spleens on average (Fig. 6C). Importantly, 2- to 4-fold-higher levels of expansion of $Ly5.1^+$ $CD8^+$ T cells were observed in mice that received the ΔAC toxoids B18 ($P < 0.01$) and A+C18 ($P < 0.05$) than in mice receiving 0.3 nmol of the nontranslocating 233B-KP/AC^- toxoid or 2 nmol of free synthetic $OVA_{257-264}$ peptide with poly(I·C). These results show that B18 and the A+C18 toxoid, which had the AC domain replaced by an artificial polypeptide sequence, were still able to deliver the $OVA_{257-264}$ epitope into the cytosolic antigen presentation pathway of APCs *in vivo*, thus priming a strong $OVA_{257-264}$-specific $CD8^+$ T cell immune response in mice.

It is noteworthy that despite being impaired in translocation of the $OVA_{257-264}$ epitope into cytosol of APCs for presentation *in vitro*, the control 233B-KP/AC^- toxoid still exhibited a low but statistically significant capacity to stimulate proliferation of OT-I cells *in vivo*, compared to the PBS control and immunization with mock toxoid ($P < 0.01$). This indicates that the capacity of the 233B-KP/AC^- toxoid to specifically bind to $CD11b^+$ APCs enabled sufficient levels of cross presentation to promote induction of detectable $CD8^+$ T cell response *in vivo*.

It was next important to establish the polyepitope delivery and $CD8^+$ CTL-priming capacity of the ΔAC toxoids in a clinically relevant system. To this end, advantage was taken of the facts that 50 to 85% of healthy adults are latently infected with cytomegalovirus (CMV) and that several strong and highly conserved $CD8^+$ CTL epitopes of the pp65 phosphoprotein of CMV have been identified (46, 52). Moreover, efficient tools for *ex vivo* expansion of CMV-specific $CD8^+$ cytotoxic T lymphocytes exhibit a clear clinical potential for use in the treatment of CMV disease in recipients of bone marrow transplants (15, 43). Therefore, a B+D18 construct was prepared, in which the AC domain was replaced by a synthetic 203-residue polypeptide composed of conserved $CD4^+$ and $CD8^+$ CTL epitope sequences (Table 1). In parallel, the same polyepitope was placed adjacent to the $OVA_{257-264}$ epitope in the control 108A/233D/AC^- toxoid, and it was verified that both constructs retained the capacity to deliver the $OVA_{257-264}$ epitope for presentation by APCs *in vitro* (data not shown). The capacity of the two constructs to activate and expand human pp65-specific $CD8^+$ CTLs could then be compared. As shown in Fig. 7A, incubation of PBMCs from CMV-seropositive healthy HLA.A2 donors with 5 nM B+D18 or 108A/233D/AC^- toxoids for 6 h resulted in a significant increase in the percentage of IFN-γ-secreting $CD8^+$ T cells ($P < 0.01$). Consequently, a statistically significant ($P < 0.01$) increase in overall IFN-γ production by PBMCs from seropositive donors was detected upon 18 h of stim-

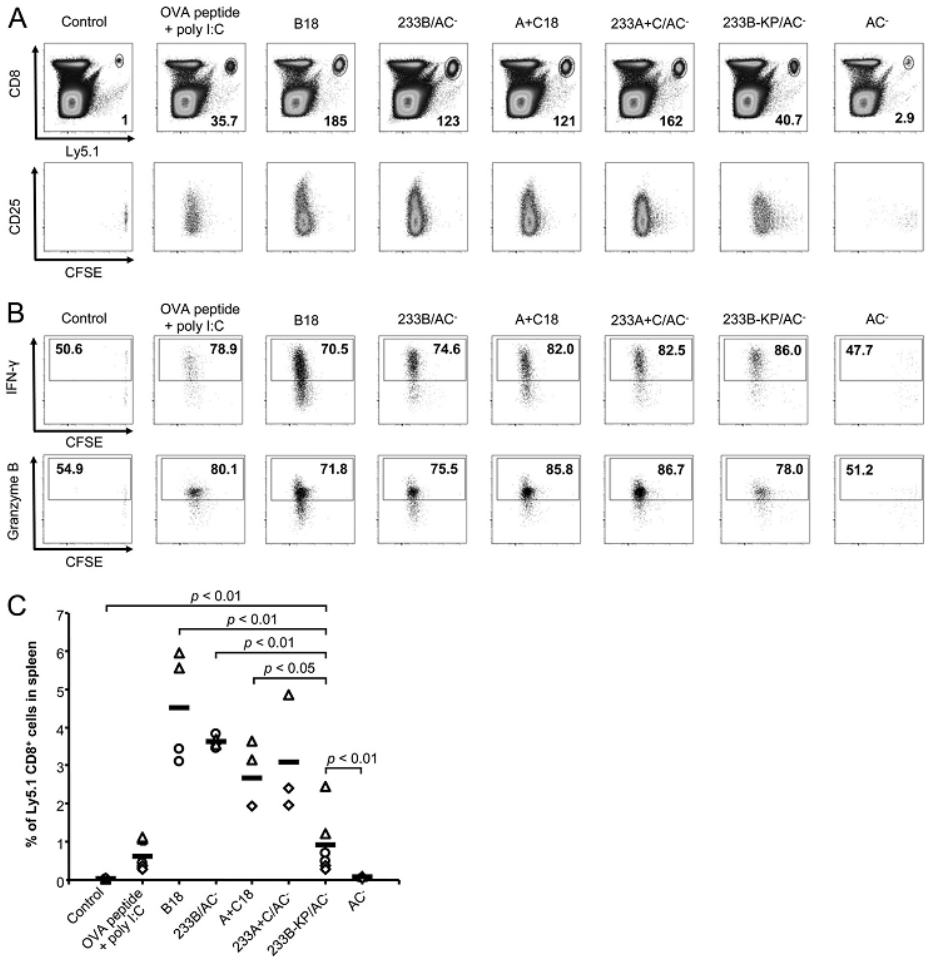

FIG 6 The CyaAΔAC toxoid delivering the $OVA_{257-264}$ epitope induces expansion of functional antigen-specific $CD8^+$ T lymphocytes *in vivo*. CFSE-labeled $CD8^+$ T cells (1.5×10^6) were purified from OT-I mice on a Ly5.1 background and injected i.v. into C57BL/6 mice (Ly5.2 background). Twenty-four hours later, the mice were immunized i.v. with the indicated toxoids (0.3 nmol) or i.p. with 2 nmol of $OVA_{257-264}$ peptide with 75 μg of poly(I-C). Control mice received PBS only. Mice were euthanized 4 days after immunization, and their spleens were analyzed by flow cytometry. (A) Expansion of $Ly5.1^+$ $CD8^+$ T cells induced by immunization with the toxoids or $OVA_{257-264}$ peptide plus poly(I-C) relative to the control is shown in bottom right corner of each dot plot. Data from one representative mouse of two immunized mice per experimental condition are shown. Data are representative of two independent experiments. (B) *In vivo*-expanded $OVA_{257-264}$-specific OT-I $CD8^+$ T cells produce IFN-γ and granzyme B. Spleen cells were incubated with 1 μM $OVA_{257-264}$ peptide for 6 h *in vitro*, and brefeldin A (1 μM) was added for the last 4 h. The percentage of $Ly5.1^+$ cells producing IFN-γ and granzyme B was determined by flow cytometry and is shown inside the positive gate in each dot plot. Data are from one representative mouse out of two mice per experimental condition and are representative of two independent experiments. (C) Percentage of $Ly5.1^+$ $CD8^+$ T cells in spleens of immunized mice. Data from repeated experiments involving two mice per group were pooled, and the horizontal bars represent the mean values. Symbols correspond to individual experiments.

ulation with B+D18 or 108A/233D/AC^- toxoids or a 118-fold-higher molar amount of the pp65 protein, used as a positive control (Fig. 7B). In contrast, no such increase in percentage of $CD8^+$ T cells secreting IFN-γ or in total secreted amounts of IFN-γ was observed for cells treated with the mock toxoids lacking the pp65 epitopes, such as the B18 protein and AC^- toxoid (Fig. 7A and B). It can hence be concluded that the ΔAC protein B+D18 delivered the inserted pp65 polyepitope for antigenic presentation by primary human APCs as efficiently as the full-length 108A/233D/AC^- toxoid and that both proteins were able to induce specific *ex vivo* stimulation of human CMV-specific $CD8^+$ T cells in cultures of PBMCs of CMV-seropositive donors with a 100-fold-higher molar efficiency than the free pp65 protein.

Therefore, we next examined the capacity of the B+D18 and

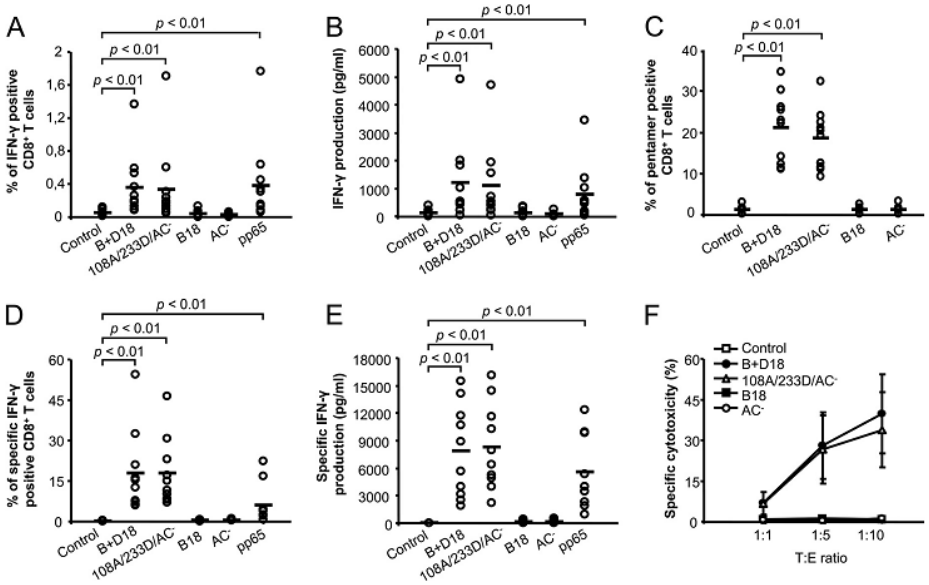

FIG 7 CyaAΔAC toxoid delivering a CMV pp65 polyepitope effectively activates and expands human CMV-specific $CD8^+$ cytotoxic T lymphocytes. (A) *Ex vivo* activation of pp65-specific $CD8^+$ T lymphocytes. PBMCs (5×10^5) from CMV-seropositive donors were stimulated *in vitro* in the presence of the indicated toxoids (5 nM) or with the pp65 protein (590 nM) for 6 h. The percentage of $CD8^+$ T cells producing IFN-γ was determined by flow cytometry. The horizontal bars represent means of pooled values obtained for PBMC samples from 10 donors. (B) Levels of IFN-γ secreted into culture supernatants by cells stimulated for 18 h as described above were determined using a cytometric bead array. (C) Clonal expansion of pp65-specific $CD8^+$ T lymphocytes in PBMC from 10 CMV-seropositive donors cultured for 7 days with 5 nM toxoids and stained with MHC I pentamers loaded with the 495-503 peptide of pp65. (D) Frequency of clonally expanded IFN-γ-producing pp65-specific $CD8^+$ T lymphocytes. Washed PBMCs cultured for 7 days in the presence of 5 nM toxoids were restimulated by coincubation with T2 cells pulsed with 10 μg/ml of CMV pp65 peptide (amino acids 495 to 503) at a 10:1 ratio for 4.5 h. Background levels of IFN-γ production resulting from restimulation with T2 cells loaded with an irrelevant HIV-1 Pol peptide were subtracted. (E) Levels of IFN-γ secreted into culture supernatants by clonally expanded pp65-specific $CD8^+$ T cells following restimulation for 9 h. (F) Clonally expanded pp65-specific $CD8^+$ T lymphocytes were coincubated at the indicated target/effector (T:E) ratios with 4×10^4 CFSE-labeled T2 cells pulsed with 10 μg/ml CMV pp65 peptide (amino acids 495 to 503). T2 cell permeabilization was determined after PI staining by FACS analysis. Background lytic activity on T2 cells pulsed with the irrelevant HIV-1 Pol peptide was subtracted. The data were obtained with PBMCs from nine CMV-seropositive donors.

108A/233D/AC^- toxoids to expand CMV-specific $CD8^+$ T cells recognizing the HLA-A*0201-restricted pp65 epitope NLVPM VATV (residues 495 to 503). As documented in Fig. 7C by comparison to mock-treated or control PBMCs, after 7 days of culture of PBMC with 5 nM B+D18 and 108A/233D/AC^- toxoids, a significant expansion of CMV pp65-specific $CD8^+$ T lymphocytes was detected using MHC I pentamers loaded with the 495 to 503 pp65 peptide. Indeed, IFN-γ-secreting $CD8^+$ T cells represented 10 to 35% of all $CD8^+$ T cells present (Fig. 7D), and this pp65-specific stimulation was accompanied by a 10-fold increase (up to 18,000 pg/ml) of levels of secreted IFN-γ (Fig. 7E). As further shown in Fig. 7F, the expanded $CD8^+$ T cells exhibited a cytotoxic capacity and permeabilized T2 target cells pulsed with the 495 to 503 peptide of pp65, compared to control cells, which were cultured with mock toxoid ($P < 0.01$). The *ex vivo* stimulation with the B+D18 and 108A/233D/AC^- toxoids therefore resulted in comparably high levels of activation and expansion of functional CMV pp65-specific human cytotoxic $CD8^+$ T lymphocytes (CTLs), which were able to specifically permeabilize T2 cells pulsed with the CMV pp65 peptide.

DISCUSSION

We show here that the AC domain of *Bordetella* adenylate cyclase toxin is entrained across the membrane of CD11b-expressing target cells as a mere passenger in a delivery step accomplished entirely by the RTX Hly moiety of the toxin (last 1,333 residues of CyaA). Unexpectedly, the deletion mapping results showed that the AC enzyme domain does not contain any segments that would play any active structural or mechanistic role in the process of AC polypeptide translocation across the cytoplasmic membrane of target cells. Indeed, the Hly moiety was also able to deliver to the cytosol two unrelated, 146- and 203-residue long artificial polypeptides extending from the N-terminal end of the Hly moiety in place of the AC domain. Delivery of the $OVA_{257-264}$ epitope into cells by CyaAΔAC constructs was, however, inhibited upon binding of the 3D1 antibody to the segment located between residues 372 and 400 of CyaA (22, 27), which links the AC domain to the Hly moiety. This linker segment was preserved in all CyaAΔAC constructs able to deliver to the cytosol the N-terminally attached artificial polypeptides, and its deletion with the first 424 residues abolished the capacity of CyaAΔAC to translocate the attached

polypeptides. Collectively, these results suggest that the segment linking the AC and Hly moieties plays a structural role and needs to be inserted across the cell membrane during the AC domain delivery.

Recently, processing of the CyaA molecule upon translocation of the AC domain across the target membrane was found to liberate the AC for action inside cells (4, 38, 49). This would suggest some resemblance of the mode of AC domain penetration into cells to the mechanism employed by A-B toxins. These typically consist of an enzymatic A component that is either noncovalently associated with the receptor-binding B moiety or linked to it by a disulfide bond that is reduced within the cytosol (7). Classical A-B toxins, however, deliver the enzymatic A subunit to the cytosol only upon receptor-mediated endocytosis. In sharp contrast, the AC domain of CyaA reaches the cytosol by direct translocation across the cytoplasmic membrane. Inhibitors of the known endocytic pathways do not interfere with translocation of the AC domain of CyaA into cells and elevation of cytosolic cAMP or with the AC domain-mediated delivery of inserted $CD8^+$ T cell epitopes into the cytosol of antigen-presenting cells (4, 14, 17, 42).

Recently, we showed that AC translocation into cells occurs in two steps, where the membrane-inserted translocation precursor of CyaA first generates a calcium-conducting path across the cell membrane (4). The incoming extracellular Ca^{2+} next activates cytosolic calpain for processing of the talin tether of CD11b/CD18 to the actin cytoskeleton, thereby mobilizing the toxin-receptor complexes for relocation into cholesterol-rich membrane lipid rafts (4). Indeed, as documented in this study, a reduced but readily detectable capacity to conduct calcium ions into CD11b-expressing J774A.1 cells was still associated with the B18 construct able to deliver the N-terminally fused $OVA_{249-284}$ polypeptide into cells. Such calcium-conducting activity is likely to also play an important role in the capacity of the toxoids to deliver the larger artificial polyepitopes (i.e., A+C18 and B+D18) into cells. This is suggested by our recent observation that toxoid-mediated influx of calcium ions into cells results in deceleration of the endocytic uptake (removal) of CyaA with cell membrane. As a result, a positive feedback loop of exacerbated cell permeabilization is initiated, where the efflux of potassium ions from permeabilized cells through toxin pores further decelerates the clathrin-mediated endocytosis of the toxin-receptor complexes (R. Fiser et al., submitted for publication).

Moreover, we recently observed that the CyaA-AC^- toxoid can promote maturation of bone marrow derived dendritic cells *in vitro*, and work in progress indicates that this is due to the capacity of the toxoid to conduct calcium ions and permeabilize antigen-presenting cells for K^+ efflux (M. Kosova, I. Adkins, R. Fiser, J. Masin, and P. Sebo, unpublished data). Such an adjuvant effect of the toxoid moiety is likely to contribute to the efficacy of induction of CTL responses to the delivered epitopes. A recent study showed that by eliciting K^+ efflux from dendritic cells, and perhaps some other CD11b-expressing phagocytes, the pore-forming activity of CyaA contributes to activation of the NALP3 inflammasome (5) and thereby to induction of innate IL-1β response, which may also be important for expansion of anti-tumor CTL responses (11).

It will hence be interesting to explore in more detail the relation between the capacity of CyaA toxoids to promote influx of calcium and efflux of potassium ions and their capacity to deliver CTL epitopes for MHC class I-restricted presentation to $CD8^+$ T lymphocytes. Enzymatically inactive but pore-forming CyaA-AC^- toxoids have been extensively used over the past 18 years for delivery of AC-inserted $CD8^+$ and $CD4^+$ T cell epitopes from viruses, mycobacteria, and tumors into the MHC class I- and II-restricted antigen presentation pathways of CD11b-expressing dendritic cells (8, 9, 21, 31, 41). Promising CyaA-derived vaccines delivering within the AC domain a defined $CD8^+$ CTL epitope from human tyrosinase or two large segments of the E7 oncoprotein of human papillomavirus 16 (HPV16) and HPV18 have entered phase I clinical studies and are being evaluated as experimental immunotherapeutic treatments for metastatic melanoma and cervical cancer, respectively. The results presented here provide the proof of concept for construction of a second generation of CyaA-based antigen delivery tools having the entire AC domain replaced by large artificial CTL polyepitope strings or entire tumor-specific antigens. This provides grounds for the design of polyvalent CTL vaccines.

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ADDENDUM IN PROOF

After our paper was published ahead of print, it was brought to our attention that the hemolysin moiety of CyaA (residues 373 to 1,706) was able to deliver across the cytoplasmic membrane of erythrocytes and into cytosol of target cells also the enzymatically active C180 fragment of the pertussis toxin subunit S1 (Iwaki M, Kamachi K, Sato H. 1998. Biological activities of the subunit of pertussis toxin: analysis of PTS1-ACT fusion. Zentbl. Bakteriol. Suppl. **29**:64–65). This work by Iwaki et al. is in line with conclusions of our study.

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6.6 PUBLICATION 5

Skopova K., Tomalova B., Kanchev I., Rossmann P., **Svedova M.**, Adkins I., Bibova I., Tomala J., Masin J., Guiso N., Osicka R., Sedlacek R., Kovar M., Sebo P. (2017) Hemolytic activity of adenylate cyclase toxin is not required for lung colonization and subversion of dendritic cell function but contributes to virulence of *Bordetella pertussis*. *Infect Immun,* in press

The bi-functional adenylate cyclase toxin-hemolysin plays a crucial role in virulence of the whooping cough agent *Bordetella pertussis*, particularly in the initiation of lung colonization. While the importance of the cAMP-elevating activity of CyaA for the colonization and immunomodulatory capacities of *B. pertussis* has previously been established, the role of the pore-forming (hemolytic) activity has not been tested. The hemolysin (Hly) moiety of CyaA forms cation-selective pores that permeabilize target cell membranes and account for the hemolytic activity of *B. pertussis* on blood agar. Here we constructed the first *B. pertussis* mutant that secretes a non-hemolytic CyaA toxin ($AC^{+}Hly^{-}$) with an intact capacity to deliver the AC enzyme and elevate cAMP in host phagocytes.

Such $AC^{+}Hly^{-}$ bacteria still skewed the TLR-triggered maturation of co-incubated mouse dendritic cells towards a tolerogenic phenotype and inhibited their antigen-presenting capacities *in vitro.* This CyaA capacity was shown to be dependent solely on the cAMP signaling of CyaA. Using the mouse respiratory challenge model we show that the pore-forming activity of CyaA is not required for mouse colonization by *B. pertussis in vivo*. However, the hemolysin activity was found to be required for the lethality of *B. pertussis* infections at high inoculation doses and it accounted for bacterial invasion across epithelial lining of bronchioles into the lung parenchyma and for triggering of neutrophil influx, thereby exacerbating the inflammation and pathology. Intriguingly, the pore-forming activity synergized with the cAMP-elevating activity in downregulation of MHC II molecule expression levels on myeloid cells that infiltrated the infected tissue, which indicates that also the pore-forming activity of CyaA contributes to immune subversion of host defenses. These results suggest that the pore-forming capacity of CyaA importantly contributes to the lung pathology resulting from *B. pertussis* infection and it appears to play a major role

in the virulence of the pathogen, most likely through harnessing of a host response that results in lethality of *B. pertussis* infection in mice.

My contribution: Generation of BMDC, infection of BMDC *in vitro*, characterization of BMDC viability and maturation and help in experiments analyzing T cell stimulation capacity of BMDC.

cAMP-elevating capacity of adenylate cyclase toxin-hemolysin is sufficient for lung colonization but not for full virulence of *Bordetella pertussis*

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Running Title: Pore-forming activity in *B. pertussis* virulence

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Abstract

The adenylate cyclase toxin-hemolysin (CyaA, ACT or AC-Hly) of *Bordetella pertussis* targets phagocytic cells expressing the complement receptor 3 (CR3, Mac-1, $\alpha_M\beta_2$ integrin or CD11b/CD18). CyaA delivers into cells an N-terminal adenylyl cyclase (AC) enzyme domain that is activated by cytosolic calmodulin and catalyzes unregulated conversion of cellular ATP into cAMP, a key second messenger subverting bactericidal activities of phagocytes. In parallel, the hemolysin (Hly) moiety of CyaA forms cation-selective hemolytic pores that permeabilize target cell membranes. We constructed the first *B. pertussis* mutant secreting a CyaA toxin having an intact capacity to deliver the AC enzyme into CD11b-expressing ($CD11b^+$) host phagocytes but impaired in formation of cell-permeabilizing pores and unable to elevate cAMP in $CD11b^-$ cells. The non-hemolytic AC^+Hly^- bacteria inhibited the antigen-presenting capacities of co-incubated mouse dendritic cells *in vitro* and skewed their TLR-triggered maturation towards a tolerogenic phenotype. The AC^+Hly^- mutant also colonized mouse lungs as efficiently as the parental AC^+Hly^+ strain. Hence, elevation of cAMP in $CD11b^-$ cells and/or the pore-forming capacity of CyaA were not required for infection of mouse airways. The latter activities were, however, required for bacterial penetration across epithelial layer, for enhanced neutrophil influx into lung parenchyma during sublethal infections and for exacerbated lung pathology and lethality of *B. pertussis* infections at higher inoculation doses (>10^7 CFU/mouse). The pore-forming activity of CyaA further synergized with the cAMP-elevating activity in downregulation of MHC II molecules on infiltrating myeloid cells, likely contributing to immune subversion of host defenses by the whooping cough agent.

Author Summary

The adenylate cyclase toxin-hemolysin (CyaA) of *Bordetella pertussis* is a sophisticated bi-functional toxin that swiftly annihilates the bactericidal functions of sentinel cells of innate immunity. It delivers into neutrophils, macrophages and other complement receptor 3-expressing ($CD11b^+$) myeloid cells an extremely active adenylyl cyclase enzyme. This then subverts cellular signaling by catalyzing massive production of the key second messenger molecule cAMP. In parallel, the hemolysin moiety of CyaA permeabilizes cellular membrane and eventually provokes lysis of cells. We have used the mouse intranasal challenge model and assessed the virulence of a set of unique *Bordetella pertussis* mutants having the invasive AC enzyme and hemolysin activities of CyaA dissociated. The study revealed that alone the

capacity of CyaA to increase cAMP concentrations in $CD11b^+$ phagocytic cells is necessary and sufficient for the pathogen to colonize host airways. The hemolysin activity or capacity to intoxicate non-myeloid cells was not required for persistent colonization of mouse lungs. The latter activities, however, accounted for virulence of *Bordetella* invasion into lung parenchyma and inflammatory damage of infected lungs. The pore-forming activity of the CyaA toxin thus likely synergizes with the AC enzyme action in manipulation of host immunity.

Introduction

The adenylate cyclase toxin-hemolysin is produced by all three *Bordetella* species pathogenic to mammals and it plays a prominent role in the early phases of respiratory tract colonization by the whooping cough agent *B. pertussis* [1,2,3]. The toxin specifically binds the CD11b subunit of the complement receptor 3 [4,5] and exerts an array of immunosubversive and cytotoxic activities on myeloid phagocytes. CyaA delivers a cell-invasive adenylyl cyclase (AC) enzyme domain into cytosol of $CD11b^+$ cells, where the AC is activated by calmodulin and converts cytosolic ATP to the signaling molecule cAMP. The generated supraphysiological levels of cAMP then near-instantly ablate bactericidal oxidative burst and opsonophagocytic killing capacities of neutrophils and macrophages [6,7,8,9,10]. With about two orders of magnitude lower efficacy, the CyaA toxin can penetrate also non-myeloid cells that lack the CR3 receptor ($CD11b^-$ cells), where due to its extremely active AC enzyme it can elevate cAMP concentrations to well detectable and physiologically relevant concentrations also in epithelial and other host cells [10,11]. Indeed, CyaA amounts detected in nasopharyngeal fluids and washes from diseased infants and infected olive baboons [12] indicate that CyaA may also be playing a prominent role in perturbation of barrier and innate immune defense functions of epithelia of airway mucosa. Recently, CyaA-bearing outer membrane vesicles (OMVs) shed by *B. pertussis* were shown to deliver CyaA into epithelial cells across their apical cell surface, through which the free secreted CyaA translocates very inefficiently [13,14]. Signaling of the OMV-delivered CyaA might thus be impairing the barrier function of polarized epithelial layers and enable access of the free toxin to the basolateral membrane of epithelial cells, through which CyaA invades epithelial cells more efficiently [14].

The CyaA protein consists of an N-terminal enzymatic AC domain (~400 residues) that is fused to a pore-forming Repeat-in-ToXin (RTX) family hemolysin/cytolysin (Hly) moiety of about 1300 residues [15]. The Hly comprises a hydrophobic pore-forming domain (residues 500-700), a fatty acyl-modified domain (residues 800-1000), a vast calcium-binding domain characteristic of RTX proteins (residues 1000-1600) and a C-terminal secretion signal [15,16]. The cytotoxic activities of CyaA strictly depend on posttranslational activation of pro-CyaA by covalent palmitoylation and on functional folding of the RTX domain upon loading of ~40 calcium ions into RTX binding sites [15,16,17,18,19,20,21]. The Hly moiety then mediates CyaA binding to CR3 [22] and enables delivery of the AC enzyme domain into the cytosol of host cells [23,24,25]. In parallel, the Hly forms small cation-selective pores that

permeabilize the cytoplasmic membrane of target cells and account for the moderate hemolytic activity of CyaA on erythrocytes [25,26]. Cell permeabilization by Hly also elicits efflux of cytosolic K^+ ions and activates the p38 and JNK kinases [27], thus contributing to TLR-induced NALP3 inflammasome formation and IL-1β secretion by dendritic cells [28]. The pore-forming activity of CyaA further synergizes with signaling of cAMP produced by the invasive AC enzyme in bringing about the overall cytotoxic (cytolytic) potency of CyaA [29,30].

Upon initial interaction with N-linked oligosaccharides [31,32], CyaA specifically binds a loop outside of the I-domain of the CD11b subunit of the $\alpha_M\beta_2$ integrin (CR3) [5] and the toxin delivers the AC enzyme into phagocyte cytosol in two steps [33]. Compelling indirect evidence suggests that the AC-translocating and pore-forming activities of CyaA are mutually independent and are accomplished by two distinct subpopulations of CyaA conformers that employ the same transmembrane CyaA segments in an alternative manner [34]. The balance between the two toxin activities can, indeed, be shifted in either direction by specific residue substitutions [35,36,37,38]. Recently, we combined a glutamine (E570Q) substitution of the glutamate residue 570 (within the pore-forming domain) with an arginine substitution of the lysine 860 residue (K860R) in the second acylation site of the toxin [29,39]. This yielded a non-hemolytic (AC^+Hly^-) CyaA-E570Q+K860R construct [37] that retains a full capacity to deliver the AC enzyme and elevate cAMP concentrations in CD11b-expressing ($CD11b^+$) myeloid cells. Its capacity to form membrane pores and permeabilize $CD11b^+$ cells is, however, largely impaired [37]. Further, also the residual capacity of CyaA to penetrate $CD11b^-$ cells is strongly decreased. Compared to intact CyaA, the CyaA-E570Q+K860R protein exhibits an about 20-fold reduced specific capacity to deliver the AC enzyme into erythrocytes and its specific capacity to permeabilize and lyze erythrocytes is reduced by almost two orders of magnitude [37]. The AC^+Hly^- toxin hence elevates cAMP concentrations in $CD11b^+$ myeloid phagocytes without permeabilizing them any importantly and this non-hemolytic AC toxin is almost inactive on non-myeloid $CD11b^-$ cells devoid of the CR3 receptor, such as erythrocytes or airway epithelial cells.

The capacity of CyaA to increase cAMP concentrations in host cells was previously shown to be essential for the capacity of *B. pertussis* to colonize mouse lungs [2,3,40]. It remained, however, unclear if this was solely due to cAMP elevation in phagocytes or whether the pore-forming activity of CyaA also plays a role in bacterial virulence. This issue could not be addressed before, as all previously used non-hemolytic *B. pertussis* mutants were producing CyaA variants with deletions in the pore-forming domain and such toxoids are

unable to translocate the AC enzyme into cells to raise cellular cAMP levels [3,41,42]. Absence of this crucial immunomodulatory action of CyaA on $CD11b^+$ phagocytes hence confounded the outcome of the animal challenge studies and all previously used non-hemolytic *B. pertussis* mutants scored as unable to colonize mouse lungs any efficiently [2,3,40,41,42]. Here we show that neither the pore-forming (hemolytic) activity of CyaA toxin on $CD11b^+$ phagocytes, nor its capacity to elevate cAMP in $CD11b^-$ cells are *per se* required for persistent sublethal colonization of mouse lungs by *B. pertussis*. These capacities, however, account for *B. pertussis* translocation into lung parenchyma and for provoking enhanced neutrophil infiltration and inflammatory damage of infected tissue.

Results

cAMP-elevating activity of CyaA is sufficient for immunomodulatory shaping of dendritic cell phenotype by *Bordetella pertussis*.

We first constructed an AC^+Hly^- *B. pertussis* mutant that secreted the cell-invasive but non-hemolytic CyaA-E570Q+K860R toxin [37]. The AC^+Hly^- mutant was unable to produce any hemolytic halos around bacterial colonies on BG agar plates (Fig. 1A), while it produced normal levels of PT, PRN and FHA virulence factors (data not shown). AC^+Hly^- *B. pertussis* also secreted the same amounts of the CyaA protein as the parental AC^+Hly^+ *B. pertussis* Tohama I strain (Fig. 1B). As shown in Fig. 1C, combination of the E570Q and K860R substitutions strongly reduced the specific capacity of the AC^+Hly^- CyaA to bind sheep erythrocytes (RBC) and ablated its capacity to translocate the AC domain into these model $CD11b^-$ cells. In contrast, the AC^+Hly^- toxin still elicited equally high cAMP concentrations in CD11b-expressing bone marrow-derived DCs as the parental AC^+Hly^+ protein (Fig. 1D). The AC^+Hly^- toxin-producing *B. pertussis* mutant was thus fully capable to elevate cAMP levels in CR3-expressing ($CD11b^+$) myeloid cells.

It has previously been shown that CyaA-elicited signaling of the cAMP-activated protein kinase A modulates maturation and cytokine production of LPS-stimulated DCs [52,53,54,55,56,57,58]. It remained, however, unclear if the cell-permeabilizing capacity of CyaA modulates the cAMP-mediated skewing of the phenotypic maturation of DCs exposed to live *B. pertussis*. We thus compared expression of maturation markers, profiles of secreted cytokines and the antigen presenting capacity of DCs that were co-incubated *in vitro* with live AC^+Hly^+, AC^+Hly^- and the AC^-Hly^+ *B. pertussis* cells at an MOI of 100:1. The AC^-Hly^+ *B. pertussis* strain was used as negative control, as it secretes the CyaA-AC^- toxoid having the AC enzyme activity ablated by insertion of a GlySer dipeptide between residues 188 and 189 of the AC domain [59]. As a further control for CR3 engagement effects, the mutant producing a doubly detoxified (non-enzymatic and non-hemolytic) AC^-Hly^- toxoid was used. Bacteria killed by heating at 70°C for 30 min (HI-Bp) were then used to control for unspecific effects of bacteria-shed TLR ligands, such as LPS and other bacterial components unrelated to CyaA.

DCs were exposed to live bacteria for 1 h before kanamycin (100 µg/ml) was added to kill the extracellular bacteria and the incubation was continued for additional 23 h to allow DC maturation. When exposed to bacteria producing enzymatically active AC toxin (AC^+Hly^+

and AC^+Hly^-), capable to elevate intracellular cAMP concentrations, the DCs exhibited equal or even higher viability at 4 h post as DCs exposed to heat-killed (HI-Bp) bacteria or the live AC^-Hly^+ mutant (Fig. 2A, *c.f.* Fig. 1C). At 24 hours, however, the expected cAMP signaling provoked drop of survival rate of DCs treated with the AC^+ bacteria was already observed (data not shown). Following continued incubation for 24 h with kanamycin-killed bacteria as a source of TLR ligands, a comparable stimulation of expression of most of the maturation markers was observed with DCs pre-incubated with heat-inactivated bacteria as upon exposure to bacteria producing the AC^- toxoids (Fig. 2B). However, a specifically reduced expression of the CD54 marker and notably of the CD40 co-stimulatory molecule was observed with DCs that were exposed to the parental AC^+Hly^+ strain and the non-hemolytic AC^+Hly^- mutant that both produce enzymatically active CyaA proteins that elevate cAMP in DCs (*c.f.* Fig. 1D). Hence, the interference of CyaA activity with DC maturation was entirely cAMP-dependent and did not involve the cell-permeabilizing (hemolytic) capacity of the secreted CyaA.

Similarly, when cytokine profiles of DCs were examined (Fig. 2C) the DCs incubated with AC^-Hly^+ and AC^-Hly^- mutants exhibited a similar profile of cytokine production as cells incubated with heat-inactivated bacteria (primarily LPS-stimulated). In contrast, a clear inhibition of TLR ligand-induced production of the pro-inflammatory cytokines TNF-α and IL-12, as well as an enhanced secretion of the immunosuppressive cytokine IL-10 by DCs was observed upon pre-incubation with the AC^+Hly^+ and AC^+Hly^- bacteria expressing the enzymatically active CyaAs.

Furthermore, exposure of DCs for 4 h to the non-hemolytic (AC^+Hly^-) mutant, or to the parental (AC^+Hly^+) strain, modulated to similar extent the OVA antigen-presenting capacity of the DCs co-incubated with OVA-specific $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T lymphocytes (Fig. 2D). Compared to treatment with heat-killed bacteria, the co-incubation of DCs with bacteria expressing the AC^- toxoids enhanced the induction of OVA-specific IFN-γ secretion by OVA-specific $CD4^+$ T cells from OT-II mice, while OVA presentation by DCs exposed to AC^+Hly^+ or AC^+Hly^- bacteria yielded a reduced capacity to stimulate IFN-γ secretion by OVA-specific $CD4^+$ T cells. In contrast, DCs exposed to AC^+Hly^+ or AC^+Hly^- bacteria exhibited clearly enhanced OVA-dependent production of IL-10 and IL-17A, as compared to DCs exposed to heat-killed cells or the bacteria producing the AC^- toxoid. DCs incubation with the parental AC^+Hly^+ or AC^+Hly^- bacteria enhanced also OVA-dependent production of the IL-10 and IL-17A cytokines by $CD8^+$ OT-I T lymphocytes. As further shown in Fig. 2E, exposure of DCs to both AC^+ strains (Hly^+ or Hly^-), but not the incubation

with the two AC^- mutants, strikingly enhanced the capacity of DCs to promote expansion of antigen-specific $CD4^+CD25^+Foxp3^+$ regulatory T cells. The pore-forming (hemolytic) activity of CyaA thus did not counteract the tolerogenic skewing of DC phenotype triggered by the cAMP-elevating CyaA toxin secreted by *B. pertussis*.

The sole capacity of CyaA to elevate cAMP in CD11b-expressing cells enables mouse lung colonization by *B. pertussis*.

We next assessed if the specific capacity of CyaA to elevate cAMP in CR3-expressing cells was sufficient, or whether the cell-permeabilizing and $CD11b^-$ cell-intoxicating activities of CyaA were also required for virulence of *B. pertussis* in the intranasal infection model in mice. In total, a series of eight colonization experiments was performed in outbred Swiss CD-1 mice with the parental AC^+Hly^+ strain and seven and six experiments were respectively performed with the AC^-Hly^+ and AC^+Hly^- *B. pertussis* mutants. The animals were challenged with doses ranging from 10^4 to 5 x 10^6 CFU/mouse and irrespective of the used challenge dose, the experiments yielded consistent colonization trends. A representative result of one experiment performed with the challenge dose of 1 x 10^5 CFU/mice is shown in Fig. 3A. The non-hemolytic AC^+Hly^- mutant colonized mice as efficiently as the parental AC^+Hly^+ strain, exhibiting consistent increase in CFU counts in the lungs of infected mice by at least an order of magnitude at the maximal colonization level on day 8 post challenge. In contrast, the AC^-Hly^+ mutant proliferated in the lungs by about a half order of magnitude only, with CFU counts peaking already on day 5 and progressively dropping on days 8 and 12. Hence, the capacity to deliver an active AC enzyme and elevate cAMP in $CD11b^+$ cells was sufficient to support the capacity of *B. pertussis* to multiply and persist in mouse lungs, while the capacities to form hemolytic pores and/or act on $CD11b^-$ cells were dispensable.

However, the AC^+Hly^- mutant exhibited a clearly decreased virulence when administered at increased challenge doses used for determination of the LD_{50} value. As shown by a representative result in Fig. 3B, up to 80 % of the mice challenged with 10^8 CFU of the parental AC^+Hly^+ strain died within 2 days from intranasal infection and the remaining infected mice succumbed by day 4, yielding an LD_{50} value of 4.1 ± 1.7 x 10^7 CFU/mouse. In contrast, no mice died upon infection with 10^8 CFU of the non-hemolytic AC^+Hly^- mutant. When a higher inoculation dose of 6 x 10^8 CFU/mice of the AC^+Hly^- mutant was used (Fig. 3C), the mice were dying only on days 8 to 9, hence 6 days later then mice infected by the lower dose of the parental AC^+Hly^+ strain. At the level of statistical significance of $p < 0.05$,

an about an order of magnitude increased LD_{50} value of 3.5 ± 0.7 x 10^8 CFU was determined for the AC^+Hly^- mutant. This represents, however, an overestimation of the real virulence of the AC^+Hly^- mutant, as the LD_{50} value does not take into account the importantly delayed onset of mice death (by almost a week). The AC^+Hly^- mutant was, however, still importantly more virulent that the AC^-Hly^+ mutant that did not cause any lethality at all, even at inoculation doses exceeding 4.3 x 10^8 CFU/mouse ($p < 0.001$).

AC^+Hly^- mutant colonization elicits lower levels of lung inflammation.

To determine the extent of lung pathology produced by the three bacterial strains, mice were infected with 10^5 CFU of each strain. Animals were sacrificed on day 6 and pathological lesions were assessed by two independent examining pathologists in parallel and in a blinded manner, without revealing them the identity of the samples.

Using samples from 4 animals per group, striking differences in lung pathology provoked by the three strains were observed (Fig. 4A). The lungs of control mice, inoculated with sterile SS medium exhibited unaltered microscopical structure with delicate continuous alveolar septa, patent alveoli, well preserved bronchi and bronchioles and intact uniform respiratory epithelium. In sharp contrast, inoculation of mice with 10^5 CFU of the parental AC^+Hly^+ *B. pertussis* strain resulted in prominent broncho-pneumonic lesions (Fig. 4A). These affected about ~10% of the lung area (Fig. 4B) and comprised involvement of the peribronchial parenchyma. The infiltrates consisted mainly of polymorphonuclear (neutrophilic) granulocytes, of increased numbers of monocytes/macrophages, of scattered lymphocytes and of swollen alveolar cells. The infected bronchial respiratory epithelium exhibited a typical focal to discrete hypertrophy and prominent lobar, lobular and septal congestion of the swollen pulmonary regions.

At a comparable level of bacterial load (*c.f.* Fig. 3A), the infection by the AC^+Hly^- mutant caused a distinctly milder pathology (Fig. 4A), with alveoli being generally well-preserved, except for some rare focal subpleural alveolar pneumonic foci. The level of respiratory epithelium damage seen in animals infected by the AC^+Hly^+ strain was not observed in animals infected by the non-hemolytic AC^+Hly^- mutant, which provoked markedly milder lesions that occurred mainly in form of irregular septal thickening and peribronchial inflammation. The alveolar structure remained, however, largely preserved and no prominent damage to the pulmonary parenchyma was observed. An evident peribronchial inflammatory infiltrate was present, extending to the vicinity of the bronchus i.e. the

peribronchial space, but it rarely affected the adjacent alveoli. Some lung congestion was also observed, but it was less pronounced than in the lungs infected by the parental AC^+Hly^+ strain (*c.f.* Fig. 4A).

Infection with the hemolytic AC^-Hly^+ bacteria still resulted in mild peribronchial inflammation despite of reduced bacterial colonization than by the AC^+ strains (*c.f.* Fig. 3A). Moreover, infection with the AC^-Hly^+ bacteria reproducibly caused more pronounced lung congestion than infection by the AC^+Hly^- mutant, while the small perivascular and peribronchial clusters of lymphocytes in AC^-Hly^+-infected lungs matched those seen in control mice.

When the overall involvement of the tissue was quantified by a median ratio of inflamed to total area of the lung section, a value of 0.091±0.024 (p<=0.0005) was determined for the bronchi and parenchyma of lungs infected by the AC^+Hly^+ strain (Fig. 4B). Significantly lower ratios of 0.021±0.081, (p<0.005) and 0.016±0.041, (p<0.0005) were then obtained for animals infected by the AC^+Hly^- and AC^-Hly^+ strains. Hence, at comparable levels of lung colonization (CFU/lung), the non-hemolytic AC^+Hly^- mutant provoked a significantly milder lung inflammation than the parental AC^+Hly^+ strain.

Moreover, a striking difference in location of the AC^+Hly^+ and AC^+Hly^- bacteria was observed (Fig. 5). Massive infiltration of bacteria into parenchyma was observed by immunohistological staining in the lungs infected with the wild-type AC^+Hly^+ *B. pertussis* strain that provoked formation of bacterial foci and focal pneumonia in the parenchyma (Fig. 5). In contrast, the non-hemolytic AC^+Hly^- bacteria were detected almost exclusively as attached to the epithelial cells in the bronchial lumen and were only rarely observed within the underlying parenchymal tissue.

Cell-permeabilizing activity of CyaA contributes to neutrophil recruitment into infected lungs

Previous work suggested that the cell-permeabilizing activity of CyaA contributed to NALP3 inflammasome activation and pro-inflammatory IL-1β cytokine secretion by TLR-activated DCs [28]. As IL-1β is known to induce production of neutrophil chemokines by non-immune cells in infected tissues [60], we enumerated the infiltrating neutrophil and macrophage cells in the sections of infected lungs upon NASDCL staining for neutrophils and following immunostaining for the F4/80 macrophage marker (Fig. 6A). A strongly increased number of neutrophils (0.50±0.067/350 μm^2, p<0.0001) was, indeed, observed in the inflamed parenchyma of lungs infected by the parental hemolytic AC^+Hly^+ strain (Fig. 6B), compared

to lungs colonized by similar counts of the non-hemolytic AC^+Hly^- mutant (neutrophil count 0.095±0.027/350 µm^2, p<0.005). Moreover, despite lower numbers of the AC^-Hly^+ bacteria in mice lungs on day 6 of infection, the hemolytic and cAMP not-elevating mutant still provoked a higher neutrophil infiltration into lung parenchyma (0.22±0.034/350 µm^2, p<0.005) than the more efficiently colonizing non-hemolytic AC^+Hly^- mutant (Fig. 6B), suggesting that the cell-permeabilizing activity of CyaA played a specific role in neutrophil attraction

To characterize in more detail the subpopulations of myeloid cells that infiltrated the infected lungs, immunostaining and multicolor flow cytometry was performed. Single cell suspensions were prepared from non-perfused lungs on day 6 of infection and the relative counts of various myeloid cell subsets were analyzed using the gating strategy outlined in the Supplementary Fig. 1 and 2. Dot plots obtained for one representative lung suspension for each infected mice group are shown in Fig. 6C and the average numbers of various myeloid cells per one million of all lung cells and 5 mice per group are given in Fig. 6D. In agreement with the enumeration following NASDCL staining, the colonization by the parental AC^+Hly^+ strain yielded a significantly higher recruitment of neutrophils into lung tissue than the colonization by comparable CFU amounts of the non-hemolytic AC^+Hly^- bacteria. While infection by the AC^+Hly^- mutant caused a doubling of relative neutrophil counts compared to control (~3% *versus* °~1.5% of all lung suspension cells, Fig. 6E), colonization by the AC^+Hly^+ strain provoked more than a tripling of neutrophil counts in the lungs on day 6 of infection (~7% of all cells in the lung suspensions being neutrophils). As already indicated by the histological examination, despite an impaired colonization capacity, the hemolytic AC^-Hly^+ infection still elicited higher neutrophil infiltration than the infection by the better colonizing but non-hemolytic AC^+Hly^- mutant (*c.f.* also Fig. 6B). This was not restricted to the infected lungs only. A systemic impact was also observed, with the relative counts of neutrophils in spleens of mice infected with the hemolytic AC^+Hly^+ and AC^-Hly^+ bacteria being about 2 to 3 times increased in comparison to spleens of control mice (Suppl. Fig. 5).

Differences in relative counts of eosinophils (EOS), macrophages (Mϕ), $Ly6C^{int}$ monocytes ($Ly6C^{int}$ MONO) and conventional dendritic cells (cDC) in the lungs infected by the various strains were mostly insignificant (*c.f.* Fig. 6E). There was also a discordance in macrophage infiltration levels as determined by histochemical analysis of manually selected segments of inflamed lung tissue (Fig. 6B) and by flow cytometry of total lung suspensions (Fig. 6C,D). The latter yielded a higher relative macrophage count for suspensions from AC^-Hly^+-infected lungs than for suspensions from the AC^+Hly^- sample, while the histology analysis of specific inflamed lung tissue regions yielded an opposite order of macrophage

counts. This may be explained by enhanced counts of chemo-attracted macrophages that remained in the vasculature of the $AC^{+}Hly^{-}$-infected lungs that were not perfused prior to preparation of cellular suspensions for cytometry. In contrast, in the histochemical analysis only cells that infiltrated into the inflamed parenchyma regions were counted.

The highest total counts of macrophage, $Ly6C^{high}$ monocytes and of cDCs and plasmacytoid dendritic cells (pDCs) were detected in the lungs of mice colonized by the parental $AC^{+}Hly^{+}$ strain (*c.f.* Fig. 6D). Intriguingly, these cells exhibited reduced mean levels of the MHC II molecule on cell surface, as compared to cells of the same type in the lungs infected by the $AC^{-}Hly^{+}$ and $AC^{+}Hly^{+}$ mutants that are deficient in one of the two CyaA activities (Fig. 7). Hence, the pore-forming (cell-permeabilizing) and the cAMP-elevating activities of the toxin synergized in provoking increased infiltration of MHC II molecule-expressing cells into infected lung tissue and the pore-forming activity contributed through an as yet unknown mechanism to reduction of the mean expression level of the MHC II molecules on the incoming MHC II^{+} myeloid cells. This effect of the cell-permeabilizing activity of CyaA manifested, however, only *in situ* at the site of infection and no decrease of the mean MHC II expression levels was observed for myeloid cell populations in spleens (Suppl. Fig. 6).

Discussion

We used here a *B. pertussis* strain secreting a unique AC^+Hly^- CyaA toxin construct that has the cell-permeabilizing capacity impaired and is essentially unable to bind and intoxicate non-myeloid ($CD11b^-$) cells. Mice infections with the AC^+Hly^- *B. pertussis* mutant revealed that the mere capacity of CyaA to elevate cAMP concentrations in CR3-expressing myeloid ($CD11b^+$) cells enables the pathogen to colonize host airways. At comparable counts in the lungs, the non-hemolytic AC^+Hly^- bacteria elicited much milder pathology than colonization by the parental AC^+Hly^+ strain. Hence, the pore-forming (hemolysin) activity of CyaA and/or its capacity to elevate cAMP in non-myeloid cells, such as airway epithelial cells, accounted individually or in combination for the exacerbated pathology resulting from *B. pertussis* infection. Moreover, the non-hemolytic AC^+Hly^- mutant was only found attached to the epithelial cells of the bronchial lumen, while the parental AC^+Hly^+ *B. pertussis* bacteria were able to cross the epithelial lining and infiltrated the lung parenchyma, provoking formation of pneumonic foci. Thus, the impaired hemolytic (pore-forming) activity and/or the reduced capacity of the secreted CyaA-E570Q+K860R toxin to elevate cAMP in epithelial cells, individually or in combination, accounted for the reduced tissue invasiveness and virulence of the AC^+Hly^- mutant. Compared to parental AC^+Hly^+ *B. pertussis*, more than an order of magnitude higher inoculation dose of the AC^+Hly^- mutant was, indeed, required for provoking death of infected mice, which still occurred a week later than upon infection by the AC^+Hly^+ bacteria. This suggests that the virulence and lethality of *B. pertussis* infections is largely due to a 'suicidal' hyperactivation of mouse innate defense, for which the cell-permeabilizing activity of the CyaA toxin and or cAMP intoxication of airway epithelial cells would account. The cell-permeabilizing and cell-invasive AC enzyme activities of CyaA synergized in provoking neutrophil recruitment and inflammatory damage of infected lung tissue, which could be underlined by at least three already known mechanisms. Firstly, CyaA-mediated elevation of cAMP concentration in tracheal epithelial cells was previously shown to elicit production of the IL-6 cytokine, which is known to activate cytotoxic action of neutrophils at the site of infection [2,40,61]. Secondly, cAMP signaling of CyaA was shown to induce COX-2 expression in macrophages and this would yield release of prostaglandins that serve in chemo-attraction of neutrophils [62]. The third mechanism would involve permeabilization of cells by CyaA pores and potassium efflux from cells, thus enhancing assembly of the NALP3 inflammasome in TLR-primed intraepithelial dendritic cells (DCs).

This was shown to trigger activation of caspase-1 and secretion of the pro-inflammatory cytokine IL-1β [28], which triggers neutrophil chemokine production by non-immune bystander cells [60]. Potentiation of neutrophil infiltration into lung parenchyma by these parallel mechanisms of CyaA action would then contribute the observed extent of inflammatory damage of *B.pertussis* infected lungs.

It is worth mentioning that the here-observed colonization defect of the AC^-Hly^+ mutant was much less pronounced than reported earlier [3]. In the earlier work, Khelef and co-workers used a high challenge dose of $10^{6.5}$ – 10^7CFU of the AC^-Hly^+ *B. pertussis* mutant, while we used only 10^5 CFU/mouse. It can be plausibly expected that at the higher bacterial inoculation dose an excessive harnessing of the innate host response by bacteria-shed TLR ligands would occur and may provoke enhanced infiltration of neutrophils into the infected tissue. This would likely enable rapid elimination of the AC^-Hly^+ bacteria that are unable to paralyze phagocytes by elevation of cAMP. In contrast, at the low inoculation dose of 10^5 CFU/mouse used here, the AC^-Hly^+ bacteria were able to proliferate to some extent over the first 5 days of infection and before being progressively cleared (*c.f.* Fig. 3A). Unlike the parental AC^+Hly^+ *B. pertussis*, however, the AC^-Hly^+ bacteria were not completely eliminated from mouse lungs for up to 30 days and continued to persist in the lungs at low but detectable levels of about 10^3 CFU/lung (data not shown).

The non-hemolytic AC^+Hly^- mutant then colonized mouse lungs almost as efficiently as the parental AC^+Hly^+ *B. pertussis*. This goes well with the previously reported effects of CyaA action on sentinel cells of innate immunity, where the produced cAMP signaling swiftly disarms neutrophils, promotes apoptosis of alveolar macrophages and subverts the TLR ligand-induced dendritic cell maturation and cytokine secretion [52,53,54,55,56,57,58]. We confirmed here that *B. pertussis*-produced CyaA suppresses IL-12 and TNF-α release and enhances secretion of immunosuppressive IL-10 by mouse dendritic cells that are in contact with live *B. pertussis* bacteria. The use of the AC^+Hly^- mutant further allowed to show *in vitro* that the pore-forming activity of CyaA was not contributing to the immunosuppressive shaping of DCs, which was entirely due to the capacity of CyaA to elevate cytosolic cAMP in DCs (*c.f.* Fig. 2C). The latter interfered with presentation of the OVA antigen on DCs exposed to live *B. pertussis* bacteria and reduced the antigen-specific IFNγ secretion by co-incubated T cells. At the same time, the *in vitro* action of the *B. pertussis*-secreted CyaA yielded enhanced antigen-specific secretion of IL-17A and IL-10 by co-incubated antigen-specific $CD8^+$ and $CD4^+$ T lymphocytes (*c.f.* Fig. 2D) and promoted expansion of antigen-specific $Foxp3^+CD25^+CD4^+$ T regulatory cells by the CyaA-hijacked DCs. These results obtained with

live bacteria, producing also other *B. pertussis* toxins (*e.g.* PT) and virulence factors (dermonecrotic toxin, adhesins, tracheal cytotoxin etc.), hence corroborate and validate our previous observations with purified recombinant CyaA. These studies showed that as little as 10 ng/ml of CyaA was able to skew the antigen-presenting capacities of LPS-activated dendritic cells towards a tolerogenic phenotype and made them expand antigen-specific $Foxp3^{+}CD25^{+}CD4^{+}$ Treg cells [63]. Higher CyaA concentrations were, indeed, recently found in nasal aspirates of infants with pertussis and in nasopharyngeal washes of *B. pertussis*-infected baboons [12]. It thus appears plausible to assume that CyaA may play a rather important role in suppression of adaptive T cell immune responses during natural infections by *B. pertussis* and that the action of CyaA may be involved in the previously observed expansion of regulatory T cells in *B. pertussis*-infected lungs *in vivo* [64].

Intriguingly, despite comparable levels of bacterial colonization, the parental $AC^{+}Hly^{+}$ *B. pertussis* provoked importantly higher infiltration of myeloid phagocytic cells into the infected lung tissue than did comparable counts of the $AC^{+}Hly^{-}$ bacteria. This indicates that the cell-permeabilizing activity of the CyaA toxin was importantly involved in attraction of neutrophil, macrophage, $Ly6C^{high}$ monocyte, conventional and plasmacytoid dendritic cells into *B. pertussis*-infected tissue. Alternatively, this cellular infiltration may also have been provoked by cAMP signaling-induced chemokine production by $CD11b^{-}$ bystander cells. This would have been only be triggered by the $AC^{+}Hly^{+}$ *B. pertussis*, but not by bacteria producing the non-hemolytic $AC^{+}Hly^{-}$ toxin unable to intoxicate CD11b- cells. We were unable to clearly distinguish at present if the much lower inflammation produced by the infection with $AC^{+}Hly^{-}$ bacteria was due to absence of phagocyte permeabilization, or if it was due the absence of cAMP elevation in $CD11b^{-}$ bystander cells, or both. Therefore, efforts are being undertaken to construct a next generation of non-hemolytic *B. pertussis* bacteria that would produce an $AC^{+}Hly^{-}$ toxin capable of elevating cAMP also in the $CD11b^{-}$ non-myeloid cells. If successful, such constructs will serve to address the mechanism by which the cell-permeabilizing activity synergized with the CyaA-produced cAMP signaling in causing reduced expression of the MHC II molecules on the myeloid cells infiltrating the infected lung tissue. This was observed for several types of antigen presenting cells, such as macrophages, monocytes, pDCs or cDCs. It is thus likely to have a non-negligible impact on the efficacy of antigen presentation to $CD4^{+}$ T helper cells in the course of *B. pertussis* infection. Such subversion of intraepithelial DC function would then be plausibly expected to hamper also adaptive responses of B lymphocytes. Delaying and restricting efficacy of the

antibody response and limiting development of the of B and T cell immune memory to *B. pertussis* antigens, may thus represent a second role of immunosubversive activity of CyaA.

Materials and Methods

Bacterial strains, growth conditions and plasmids. *Escherichia coli* XL1-Blue (Stratagene) was used for DNA manipulation and CyaA production. Bacteria were grown in Luria-Bertani (LB) medium supplemented with ampicillin (150 µg/ml). *Escherichia coli* SM10 λpir [43] was used for plasmid transfer into *B. pertussis* by bacterial conjugation. The *E. coli* SM10 λpir transformants were grown at 37°C in LB agar medium supplemented with kanamycin (60 µg/ml) and ampicillin (150 µg/ml). *B. pertussis* Tohama I strain was generously provided by Dr. Nicole Guiso from Institut Pasteur, Paris [44]. The parental strain and *B. pertussis* mutants were grown at 37°C on Bordet-Gengou agar (Difco, USA) media supplemented with 1% glycerol and 15% defibrinated sheep blood (BG) and 5% CO_2 for 72 h to visualize hemolysis. Liquid cultures were grown in modified Stainer-Scholte medium [45] (supplemented with 1 g/l of casaminoacids and 1 g/l of cyclodextrin) for 18 h at 37°C. The suicide vector pSS4245, generously provided by Dr. Scott Stibitz, was used for allelic exchange on *B. pertussis* chromosome, as described [46].

Mutagenesis of the *cyaA* gene on *B. pertussis* chromosome. Construction of the alleles for production of the CyaA-AC^- (AC^-Hly^+) toxoid and of the non-hemolytic CyaA-Hly^- (AC^+Hly^-) toxin mutant in *E. coli* was described in detail previously [37]. The respective mutated *cyaA* gene segments were recloned into exchange vector plasmid pSS4245 that contains a Str^R allele functional in *B. pertussis* but not in *E. coli*. Prior to mating, *B. pertussis* Tohama I strain was grown 4 days under modulating conditions on BG agar supplemented with 50 mM $MgSO_4$ (Bvg^- conditions) and mating with *E. coli* SM10 λpir transformed by the suicide plasmid constructs was performed on fresh BG agar plates supplemented with 10 mM $MgCl_2$ and 50 mM $MgSO_4$ for 3 h at 37°C. *B. pertussis* cells having incorporated the allelic exchange plasmid by single crossing-over into the chromosome were selected on BG agar supplemented with 50 mM $MgSO_4$ and 500 µg/ml of streptomycin, 30 µg/ml of ampicillin and 40 µg/ml of kanamycin for 5 days at 37°C. The resulting single colonies were restreaked on the same plates for additional 5 days before plating on BG agar lacking $MgSO_4$ (Bvg^+ conditions) to select for the second crossing over and excision of the allelic exchange vector. For each construct, several individual colonies were characterized for phenotypic change and the presence of introduced mutations and absence of undesired mutations was verified by sequencing of relevant portions of PCR amplified segments of the *cyaA* gene. Production of

PT, PRN, FHA and CyaA antigens was verified by Western blotting of bacterial suspensions using specific mouse antibodies [47].

Preparation of urea extracts for CyaA toxin assays. *B. pertussis* strains were grown for 72 h on Bordet-Gengou agar and subcultured in 2 ml of Stainer-Scholte medium to A600=1, collected by centrifugation (1 min, 15 000 x *g*) and resuspended in 200 µl of 8 M urea, 50 mM Tris pH 7.4 and 2 mM $CaCl_2$ (TUC buffer). The urea extracts were cleared by centrifugation and used for determination of AC and CyaA toxin activities**.**

Assay of AC activity. Adenylate cyclase enzymatic activity was measured in the presence of 1 µM calmodulin as previously described [48]. One unit of AC activity corresponds to 1 µmol of cAMP formed per min at 30°C and pH 8.0.

Binding and cell-invasive activities in RBCs. Erythrocyte binding and cell-invasive AC activities were determined as described in detail previously [25]. Briefly, sheep erythrocytes (5×10^8 cells/ml) were incubated with bacterial lysates at 37°C in TNC buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl and 2 mM $CaCl_2$). After 30 min, cell suspensions were washed in TNC buffer to remove unbound CyaA and divided in two aliquots. One aliquot was directly used to determine the amount of cell-associated AC activity (membrane-bound CyaA). The other aliquot was treated with 20 µg/ml of trypsin for 15 min at 37°C in order to inactivate the extracellular AC enzyme that did not translocate across cellular membrane. Soybean trypsin inhibitor (40 µg/ml) was then added to the mixture to stop the reaction before the samples were washed three times with 20 mM Tris-HCl at pH 7.4, 150 mM NaCl and 5 mM EDTA and used to determine the amount of cell-invasive AC enzyme activity. The activity of intact CyaA was taken as 100%.

Generation of mouse bone marrow-derived dendritic cells (DCs). Bone marrow-derived dendritic cells were generated according to the method of Lutz *et al*. [49]. Briefly, bone marrow cells were flushed from femurs and tibias of C57BL/6 mice, and cultured at 2×10^6/ml in 100-mm dishes in 10 ml of RPMI 1640 medium supplemented with 10% FCS (Life Technologies, USA), 0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 µg/ml amphotericin (Sigma-Aldrich, USA), 50 µM 2-mercaptoethanol, 1% non-essential amino acids (Biochrom, Germany), 1 mM sodium pyruvate, 2 mM glutamine and 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). Fresh medium was added on

day 3 and on day 6. Loosely attached cells were used for experiments at days 6 to 8. 70 - 80 % of cultured cells expressed CD11c (APC-anti-mouse-CD11c antibody, clone N418, eBioscience, USA) and 90 % CD11b (PE-anti-mouse-CD11b antibody, clone M1/70, BD Pharmingen, USA).

cAMP elevation capacity of CyaA in DCs. For determination of CyaA toxin activity (elevation of intracellular cAMP), BMDCs (10^6/ml) were incubated with appropriately diluted bacterial lysates (final CyaA concentration of 3 mU/ml) for 1 h at 37°C in D-MEM and the reaction was stopped by addition of 0.2% Tween-20 in 100 mM HCl. Samples were boiled for 15 min at 100°C, neutralized by addition of 150 mM unbuffered imidazol and cAMP concentrations were measured by ELISA as previously described [50]. The results represent average of values obtained in at least two independent experiments performed in duplicates.

Western blotting. Bacteria were grown in liquid Stainer-Scholte medium for 18 h at 37°C and cells from 1 ml of bacterial suspension were collected by centrifugation (10 min, 15 000 x *g*). The pellet was resuspended in 100 µl of Laemmli buffer (50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 1% β-mercaptoethanol) and dissolved for 5 min at 100°C. 10 µl of lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. CyaA was probed by the anti-RTX monoclonal antibody 9D4 (at a 1 : 3000 dilution) [51] and revealed by peroxidase-conjugated secondary antibody (at a 1 : 10 000 dilution; GE Healthcare, UK) using a chemiluminescence detection system (Thermo Fisher Scientific, USA) and an LAS-4000 imaging system instrument (Fuji, Japan).

Intranasal infection of mice. Four-week-old female BALB/c or Swiss CD-1 mice (AnLab, Janvier) were used in this study. Mice were anesthetized by i. p. injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) in saline and were inoculated intranasally with 50 µl of *B. pertussis* suspensions grown for 24 h in modified Stainer-Scholte liquid medium. To determine viable colony forming units (CFU), the suspensions were diluted and plated on BG agar plates.

All animal experiments were approved by the Animal Welfare Committee of the Institute of Microbiology of the ASCR, v. v. i., in Prague, Czech Republic. Handling of animals was performed according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National Assembly, Collection of Laws No. 149/2004, inclusive of

the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws No. 207/2004, on care and use of experimental animals.

Colonization experiments. Infected Swiss CD-1 mice were sacrificed by cervical dislocation 2 h after exposure to challenge suspension (day 0 + 2 h) and on the indicated days thereafter (day 5, 8, 12 and 21). The lungs were aseptically removed and homogenized in physiological solution with tissue grinders. Dilutions of lung homogenates were plated on BG agar plates and CFU were counted after 4 days of incubation at 37°C. Three mice per time point were used and this experiment was repeated eight times in total for the parental *B. pertussis* Tohama I strain, seven times for *B. pertussis*-$AC^{-}Hly^{+}$ mutant and six times for *B. pertussis*-$AC^{+}Hly^{-}$ mutant, using challenge doses ranging from 10^4 to 5 x 10^6 CFU, yielding very similar results and colonization courses. The average value of the results obtained with a challenge dose of 10^5 CFU is shown.

Determination of LD_{50}. For determination of the LD_{50} values, groups of mice were challenged intranasally with serially diluted bacterial suspensions and their survival was monitored over 10 days. The results expressed as % of surviving mice represent average of pooled values obtained in seven experiments with parental *B. pertussis* Tohama I strain and *B. pertussis*-$AC^{-}Hly^{+}$ mutant and three experiments with *B. pertussis*-$AC^{+}Hly^{-}$ mutant, using 6 to 8 mice per challenged group per used challenge dose and individual experiment.

Histological studies. Lung morphology was examined upon intranasal challenge of BALB/c mice with a dose of 1.5 x 10^5 CFU. Control mice were given 50 µl of Stainer-Scholte medium only. Mice were anesthetized by i. p. injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) in saline and sacrificed by cervical dislocation. The tracheas were cannulated and lungs were inflated with infusion of 4% w/v buffered formaldehyde solution into the thorax, excised and immersed in 4% w/v phosphate-buffered formaldehyde for 48h. Afterwards all samples were processed in a Leica ASP6025 tissue processor (Leica Biosystems, Nussloch, Germany), molded in paraffin and cut at 3 µm thickness. Hematoxylin and eosin (HE) staining was carried out using automated Ventana Symphony stainer (Ventana Medical Systems, Tucson, Arizona). Naphtol AS-D chloroacetate esterase (NASDCL) histochemistry staining of neutrophils was performed using a NASDCL staining kit (Sigma–Aldrich, Germany) according to manufacturer's instructions. For immunohistochemistry staining for the

macrophage marker F4/80 and for *B. pertussis* cells, the histological sections were rehydrated and retrieved in the pH 9 antigen retrieval solution (Zytomed GmbH, Berlin, Germany), incubated for 1h at room temperature (RT) with the mouse anti-F4-80 Mab (clone Cl:A3-1; Bio-Rad, CA, US), or with the rabbit anti-*B. pertussis* serum (kind gift of B. Vecerek), both at 1:1000 dilution, followed by secondary anti-mouse or anti-rabbit peroxidase conjugated polymer (Zytomed GmbH, Berlin, Germany) for 30 min at RT, respectively. The immune reaction was developed using the AEC solution (DAKO, Glostrup, Denmark) for 10 min at RT. Slides were mounted with Aquatex® mounting medium (Merck-Millipore, Darmstadt, Germany) and scanned using an AxioScan.Z1 automated slide scanner (Carl Zeiss, Göttingen Germany). Representative images were generated using the ZEN software (Carl Zeiss, Göttingen Germany). Quantification of F4/80 stained cell (macrophages) and neutrophils was performed on full slide scans where the two cell types were counted only in the inflammatory areas. The significance of the differences between groups was determined by unpaired two-tailed Student's *t* test. Differences were considered statistically significant if $p < 0.05$ (*), $p < 0.01$ (**).

Monoclonal Antibodies for cell phenotyping. The following anti-mouse mAbs were used for flow cytometry analysis: CD3-V500, CD4-V500, CD8-V500, CD45R (B220)-V500, CD45-PerCP, Ly6G-A700, Siglec-F-PE (BD Biosciences); CD45R (B220)-A488, CD11b-eF450, CD11c-eF450, Ly6C-A488, MHC II (I-A/I-E)-APC (eBioscience); and mPDCA-1-PE (Miltenyi Biotec). Nonspecific binding of cells was blocked by anti-mouse CD16/CD32 mAb (eBiosciences).

Surface staining and flow cytometry analysis. Single cell suspensions were prepared from unperfused lungs and spleens of BALB/c mice by homogenization in GentleMACS Dissociator (Miltenyi Biotech) and digestion with Collagenase D (1 mg/mL; Roche) in HBSS (Sigma-Aldrich) for 30 min at 37°C, followed by incubation with 50mM EDTA (Gibco) for 5 min, repeated homogenization and filtration through a 70 µm nylon cell strainer (BD Falcon). After RBC lysis (ACK Lysing buffer; Gibco), cells were resuspended in FACS buffer (PBS, 2% fetal calf serum (FCS; Gibco), 2mM EDTA), blocked by 10% BALB/c mouse serum and anti-mouse CD16/CD32 mAb (0.5 µg/sample; eBioscience) for 20 min on ice and stained with fluorochrome-labeled mAbs for 30 min on ice in dark. Cells were washed twice after each step in FACS buffer, fixed in BD Cytofix/Cytoperm solution (BD Biosciences) for 1 hour on ice and resuspended in FACS buffer. Flow cytometric analysis was performed on an

LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo X software (Tree Star). In case of lung samples, 200 000 of $CD45^+$ cells with excluded doublets and debris were run. The depicted data represent single cell populations after exclusion of doublets and debris and gated from $CD45^+$ population (for eosinophil population) or $CD45^+DUMP^-$ population, i.e. $CD45^+CD3^-CD4^-CD8^-B220^-$ population (for monocyte, macrophage and neutrophil population) or $CD45^+CD3^-$ population (for dendritic cell population) (Suppl. Fig. 1 and 2). In case of spleen samples, 200,000 lymphocytes were analyzed. The depicted data represent single cell populations after exclusion of doublets and debris (for eosinophil population) and cell populations gated from $DUMP^-$ population, i.e. $CD3^-CD4^-CD8^-B220^-$ population (for monocyte, macrophage and neutrophil population) or $CD3^-$ population (for dendritic cell population) (Suppl. Fig. 3 and 4). All dot plots show 10 000 representative cells for better clarity.

Detection of cytokine production in lungs and spleens. Mice were euthanized, lungs and spleens were aseptically collected and cut into pieces of 2 to 3 mm in size, transferred into RPMI 1640 medium containing 10% FCS, antibiotics (penicillin/streptomycin; Sigma-Aldrich), 2mM L-glutamin (Sigma-Aldrich), 1mM Na-pyruvate (Gibco), 10mM Hepes (Sigma-Aldrich) and 0.05mM 2-mercaptoethanol (Sigma-Aldrich), and placed for 30 min into CO_2 incubator (5% CO_2, 37°C), to reconstitute the physiological pH in the medium. Samples were then rotated on a laboratory rotator for 16 hours at 37°C before supernatants were collected and used for detection of selected cytokines. ELISA detection kits from R&D systems were used for detection of IL-1β, IL-6, IL-17, GM-CSF, MCP-1 and TNFα; ELISA detection kits from Novex were used for detection of IL-10, IL-12 and IFNγ, following manufacturer´s protocols. Samples were analyzed on a Tecan Infinite M200 Pro ELISA reader using i-Control software (Tecan).

Mice and cell lines for analysis of DC maturation. 6 - 8 weeks old C57BL/6 mice were purchased from the SPF breeding facility of the Institute of Molecular Genetics of the ASCR, v.v.i. in Prague, Czech Republic. OT-I mice are transgenic for the T cell receptor recognizing the ovalbumin (OVA) epitope $OVA_{257-264}$ in the context of $H\text{-}2K^b$. The OT-II mice bear the transgenic T cell receptor recognizing $OVA_{323-339}$ in the context of I-Ab and were a generous gift of Pavel Otahal, Institute of Molecular Genetics of the ASCR, v.v.i. in Prague, Czech Republic.

T cells were cultured in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, USA), 0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 µg/ml amphotericin (Sigma-Aldrich, USA), 50 µM 2-mercaptoethanol, 1% non-essential amino acids (Biochrom, Germany), 1 mM sodium pyruvate, 2 mM glutamine and 6.5 g/l glucose. RPMI 1640 medium without antibiotics were used for the infection experiments with *B. pertussis*.

Infection of DCs. DCs (10^6/ml) in D-MEM medium without antibiotics were exposed to the suspension of heat-killed (70°C, 30 min) parental *B. pertussis* bacteria or were infected with live suspensions of the indicated *B. pertussis* strains at a multiplicity of infection (MOI) of 100:1 (bacteria:DC). After addition of bacteria to DCs, plates were gently centrifuged (700 x *g*, 5 min) to enable bacterial attachment to DCs. After 1 h of incubation, extracellular bacteria were killed by addition of kanamycin (100 µg/ml) and incubation with DCs continued for the indicated times. Control cell suspensions were supplemented with kanamycin (100 µg/ml) as well.

Detection of DC viability. DCs (5×10^5/well) were infected as described above. After 1 h of bacterial infection at =100:1, killing of bacteria by kanamycin and 3 h of further incubation, the DCs were incubated with TMRE (40 nM; Invitrogen) for 15 min at 37°C and subsequently stained with Hoechst 33258 (0.5 µg/ml; Invitrogen, USA). Live ($TMRE^+$, Hoechst 33258^-) cells were detected by flow cytometry using FACS LSR II instrument (BD Bioscience, USA) and analyzed by flow cytometry software FlowJo version 7.2.1 (Treestar, Inc.).

Maturation of DCs. DCs (2×10^6/well) were incubated with *B. pertussis* bacteria at MOI=100:1 as outlined above for 24 h. DCs were next stained with APC-anti-mouse CD11c (clone N418) and one of the following antibodies: FITC-anti-mouse-I-A/I-E (clone M5/114.15.2), FITC-anti-mouse-CD80 (clone 16-10A1), FITC-anti-mouse-CD86 (clone GL1), FITC-anti-mouse-CD54 (clone YN1/1.7.4) from eBioscience (USA) or FITC-anti-mouse-CD40 (clone 3.23) and FITC-anti-mouse-H-2Kb (clone AF6-88.5) from BD Pharmingen (USA), respectively. The expression of cell surface markers by live $CD11c^+$ cells was detected by flow cytometry. Mean fluorescence intensity (MFI) values of samples were determined using FlowJo X software (Tree Star). The expression of surface markers of live DCs treated with HI-Bp was set to 100 %.

Cytokine production by DCs. The cell culture supernatants of DCs (2×10^6/well) were taken after 24 h of incubation with bacteria and the concentration of IL-10, IL-12p70 and TNF-α was determined using a mouse IL-10 and TNF-α ELISA MAX Standard kit (BioLegend, USA) and mouse IL-12p70 Duo Set ELISA kit (RD Systems, USA) according to the manufacturer's instructions, taking induction by HI-Bp as 100 %.

OVA-specific T cell responses and expansion of $CD4^+CD25^+Foxp3^+$ T cells. Naïve OVA-specific $CD8^+$ or $CD4^+$ T cells were isolated from lymph nodes and spleen of OT-I or OT-II transgenic mice, respectively, using magnetic cell separation (MACS) with $CD8^+$ and $CD4^+$ T cell isolation kits (Miltenyi, Germany) according to the manufacturer's instructions. DCs (5×10^4/well) were seeded in 96-well plate and co-incubated with the OVA protein (albumin from chicken egg white; Calbiochem, USA) and bacteria as outlined above. After 1 h of incubation, bacteria were killed by addition of kanamycin (100 µg/ml) and incubation of DCs was continued for another 4 h in the presence of ovalbumin (OVA). For subsequent MHC class II presentation to OT-II $CD4^+$ T cells, the OVA antigen at a concentration of 0.1 µM was used. 0.2 µM OVA was used to assess MHC class I presentation to OT-I $CD8^+$ T cells. After 4 h the DCs were washed and naïve OVA-specific $CD8^+$ or $CD4^+$ T cells were added and co-incubated with pretreated DCs. The production of IL-17, IFN-γ and IL-10 (RD Systems, USA) in cell culture supernatant after 3 days was determined by ELISA. Remaining $CD4^+$ T cells were collected and stained with APC-anti-mouse-CD3, Alexa488-anti-mouse-CD25 and PE-anti-mouse-Foxp3 (eBioscience, USA) using Fixation and Permeabilization buffers (eBioscience, USA). The expression of Foxp3 in $CD4^+CD25^+$ T cells was determined by flow cytometry.

Statistical analysis. The significance of the differences between groups was in general determined by unpaired two-tailed Student's *t* test. Differences were considered statistically significant if $p < 0.05$ (*), $p < 0.001$ (**). Where appropriate (indicated in Figure legends) the data were analyzed using ANOVA followed by Tukey test for pairwise comparison of sub-groups; *, **, and *** represent p-value <0.05, 0.01 and 0.001, respectively. Data are representative of at least two experiments.

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Figure Legends

Fig 1. The AC^+Hly^- variant of CyaA is produced at normal levels and preserves the DC-binding and cAMP-elevating activity of intact CyaA. (A) Parental AC^+Hly^+ *B. pertussis*, the non-hemolytic mutant expressing the CyaA-E570Q+K860R toxin (AC^+Hly^-) and the AC^-Hly^+ mutant producing an enzymatically inactive CyaA-AC^- toxoid were grown on Bordet-Gengou agar with 15% of defibrinated sheep blood for 5 days at 37°C. (B) Bacteria were grown in liquid Stainer-Scholte medium for 18 h at 37°C and CyaA toxin was detected on Western blots of bacterial lysates using the anti RTX 9D4 antibody. Values below the blot indicate the total AC enzyme activities of CyaA associated with the outer surface of bacterial cells grown in SS medium with 0.13 mM Ca^{2+} ions. (C) $CD11b^-$ sheep erythrocytes (5 x 10^8/ml) were incubated at 37°C with bacterial lysates (20 mU/ml). After 30 minutes aliquots were taken for determination of the cell-associated AC activity (binding) and of the AC activity internalized into erythrocytes and protected against digestion by externally added trypsin (invasive AC). Activities are expressed as percentages of intact CyaA activity and represent average values ± standard deviations from two independent determinations performed in duplicate. (D) AC domain translocation was assessed by determining the intracellular concentration of cAMP generated in cells following incubation with diluted bacterial lysates (final CyaA concentration of 3 mU/ml). The results represent average of values obtained in at least two independent experiments performed in duplicates.

Fig 2. The cAMP-elevating activity alone is sufficient for immunomodulatory shaping of dendritic cell phenotype by *B. pertussis*-secreted CyaA. Bone marrow-derived DCs were treated with suspension of heat-killed parental *B. pertussis* (HI-Bp; 70°C, 30 min) or infected with the different *B. pertussis* mutants at MOI=100:1. After 1 h of incubation, kanamycin (100 µg/ml) was added to kill the bacteria and DCs were further incubated for 3 or 23 h. (A) DCs survival after 4 h was determined by TMRE and Hoechst 33258 staining by flow cytometry. The viability ($TMRE^+$ $Hoechst\ 33258^-$) of untreated DCs (medium) was set to 100 %. (B) Expression of $H\text{-}2K^b$, I-A/I-E, CD80, CD86, CD40 and CD54 in living $CD11c^+$ DCs was determined by flow cytometry and (C) the secretion of TNF-α, IL-10 and IL-12p70 at 24 h was determined in DC culture supernatants by ELISA. Expression of maturation-associated molecules and production of cytokines by DCs treated with heat-killed bacteria was set as 100 %. Values represent the means ± SEM of n=3 (* $p < 0.05$). (D) DCs treated as indicated

above were co-incubated with 0.1 µM OVA protein for presentation on MHC class II molecules to OT-II $CD4^+$ T cells or with 0.2 µM OVA for presentation on MHC class I molecules to OT-I $CD8^+$ T cells. After 4 h of co-incubation the DCs were washed and naïve OVA-specific $CD8^+$ or $CD4^+$ T cells were added. Production of IL-17, IFN-γ and IL-10 in cell culture supernatants after 3 days was determined by ELISA. (E) The numbers of $Foxp3^+CD4^+CD25^+$ T regulatory cells were determined by flow cytometry after 3 days of co-culture. All experiments were reproduced at least 3 times and representative dot plots are shown. The graph values represent the means ± SEM (* $p < 0.05$).

Fig 3. AC enzyme activity of CyaA alone supports lung colonization by *B. pertussis*.
(A) Colonization of the mouse lungs. Four-week-old Swiss CD-1 mice were challenged intranasally with 10^5 CFU of the parental AC^+Hly^+ strain or of the AC^-Hly^+ and AC^+Hly^- mutants. The mean values and standard deviations from six mice per time point were plotted (except for day 12, which is a mean value from only three mice). The results represent average of values obtained in two experiments. (B) Survival rates of 4-week-old mice infected with 1 x 10^8 CFU of the WT and mutant *B. pertussis* strains (C) Survival rates at increased inoculation dose of 6 x 10^8 CFU. Mortality was monitored for 10 days postinfection. The results were reproduced in at least 3 experiments using 6 to 8 mice per challenged group per used challenge dose.

Fig 4. *B. pertussis* AC^+Hly^- mutant elicits importantly milder inflammation of colonized lungs than the parental strain. 4 BALB/c mice per group were infected intranasally with 1.5 x 10^5 CFU in 50 µl of suspensions of the indicated *B. pertussis* strains. The animals were sacrificed on day 6 and lungs were processed for staining with Hematoxylin and eosin (see Materials and Methods for details) and scanned. Control mice received SS medium, only. Left panels show longitudinal sections of the left lobes at the original magnification of 1,25x that are representative of 3 serial sections per lung lobe. Right panels show zooms with representative images of the bronchi and peribronchial parenchyma at 20x magnification. Lungs of animals infected by the AC^+Hly^+ strain exhibited bronchopneumonia affecting the regions primarily around the large lobar bronchi. Importantly milder inflammation is observed in the lungs of animals infected by the AC^+Hly^- and AC^-Hly^+ strains.

Fig 5. The AC^+Hly^- mutant does not penetrate into lung parenchyma. Lungs of BALB/c mice infected with 10^5 CFU of the indicated strains in 50 µl of suspensions were examined on

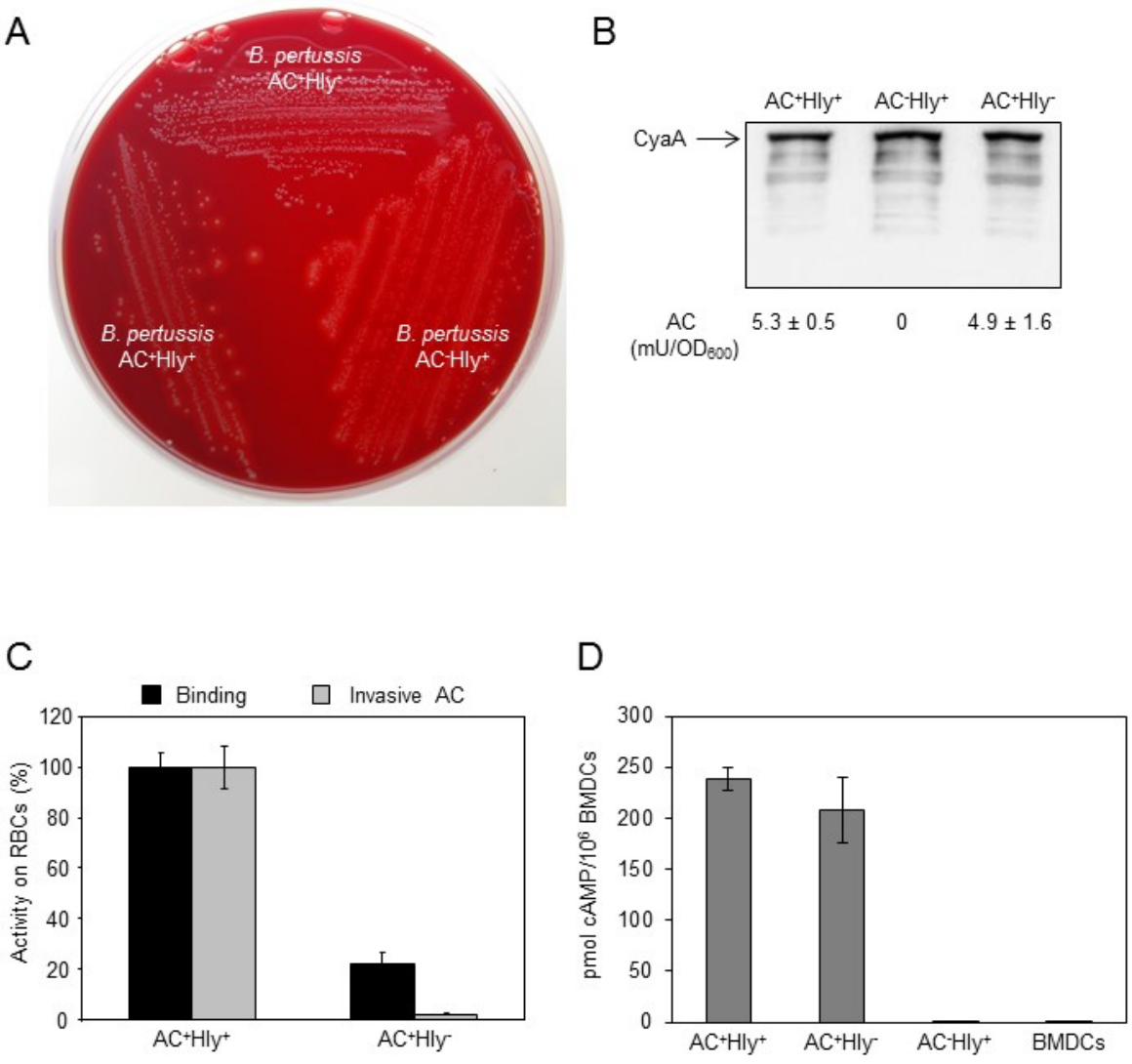
day 6 upon immunohistochemical staining for *B. pertussis* antigens with a polyclonal rabbit serum. The parental AC^+Hly^+ *B. pertussis* colonized the bronchial tree and invaded the lung alveolae and parenchyma resulting in massive inflammatory response and pneumonic foci with cellular infiltrate (right panel). The AC^+Hly^- mutant colonized the bronchial tree and was found attached to the ciliated epithelial cells of bronchi without invading the parenchyma. IHC magnifications: 1,25x (left) and 40x (right).

Fig 6. The cell-permeabilizing activity of CyaA triggers infiltration of neutrophils into *B. pertussis*-infected lungs. (A-B) BALB/c mice infected intranasally with 10^5 CFU of the indicated strains were sacrificed on day 6 and the sections of unperfused lung tissue were examined upon NASDCL histochemical and F4/80 immunohistochemical staining for neutrophils and macrophages, respectively. Representative sections documenting the main inflammatory cell components from the *B. pertussis*-induced lesions are shown. Neutrophil infiltration was significantly reduced in both AC^+Hly^- and AC^-Hly^+ strain-infected lungs, while macrophage counts were reduced only in sections of lungs infected by the AC^-Hly^+ strain. Magnification 20x. (B) Numbers of neutrophils and macrophages per surface unit were quantified by analysis of all inflamed parenchyma regions that were manually delimited on three consecutive lung sections for 4 infected animals per group (12 sections analyzed in total). C-E) Distribution of cell subsets in infected mouse lungs. BALB/c mice were infected with 1.5 x 10^5 CFU of the various infected with *B. pertussis* strains (AC^+Hly^+, AC^-Hly^+, AC^+Hly^-), using medium as control and suspensions of unperfused lungs were analyzed by flow cytometry. C) Dot plots of cell subtypes in one representative mouse lung suspension per experimental group. D) Total counts of indicated cell subsets in the infected lungs (n=5) that were not perfused prior to homogenization**.** E) Relative distribution of myeloid cell subsets in suspensions of non-perfused lungs (n=5). Groups were compared using ANOVA followed by Tukey test for pairwise comparison of sub-groups; *, **, *** represents p-value <0.05, 0.01 and 0.001, respectively. The experiment was repeated twice with similar results. EOS, eosinophils; Mϕ, macrophages; $Ly6C^{int/hi}$ MONO, monocytes; NEU, neutrophils; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells.

Fig 7. The cell-permeabilizing and AC enzyme activities of CyaA synergize in provoking reduction of MHC II molecule expression on myeloid cells in infected lungs. Expression of the MHC II molecule on selected cell subsets in lungs of BALB/c mice infected with *B. pertussis* 6 days after infection with 1.5 x 10^5 CFU of the various strains or with medium

(Control). A) Histograms for one representative mouse lung suspension per experimental group. B) Total counts of MHC II-expressing cells per one million lung cells (*n*=5). C) Mean level (MFI) of MHC II molecules detected on the surface of selected cell subsets in the infected lungs (*n*=5). Groups were compared using ANOVA followed by Tukey test for pairwise comparison of sub-groups; *, **, *** represents p-value <0.05, 0.01 and 0.001, respectively. The experiment was repeated twice with similar results. Mϕ, macrophages; Ly6C$^{int/hi}$ MONO, monocytes; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells.

Skopova *et al*. Figure 1



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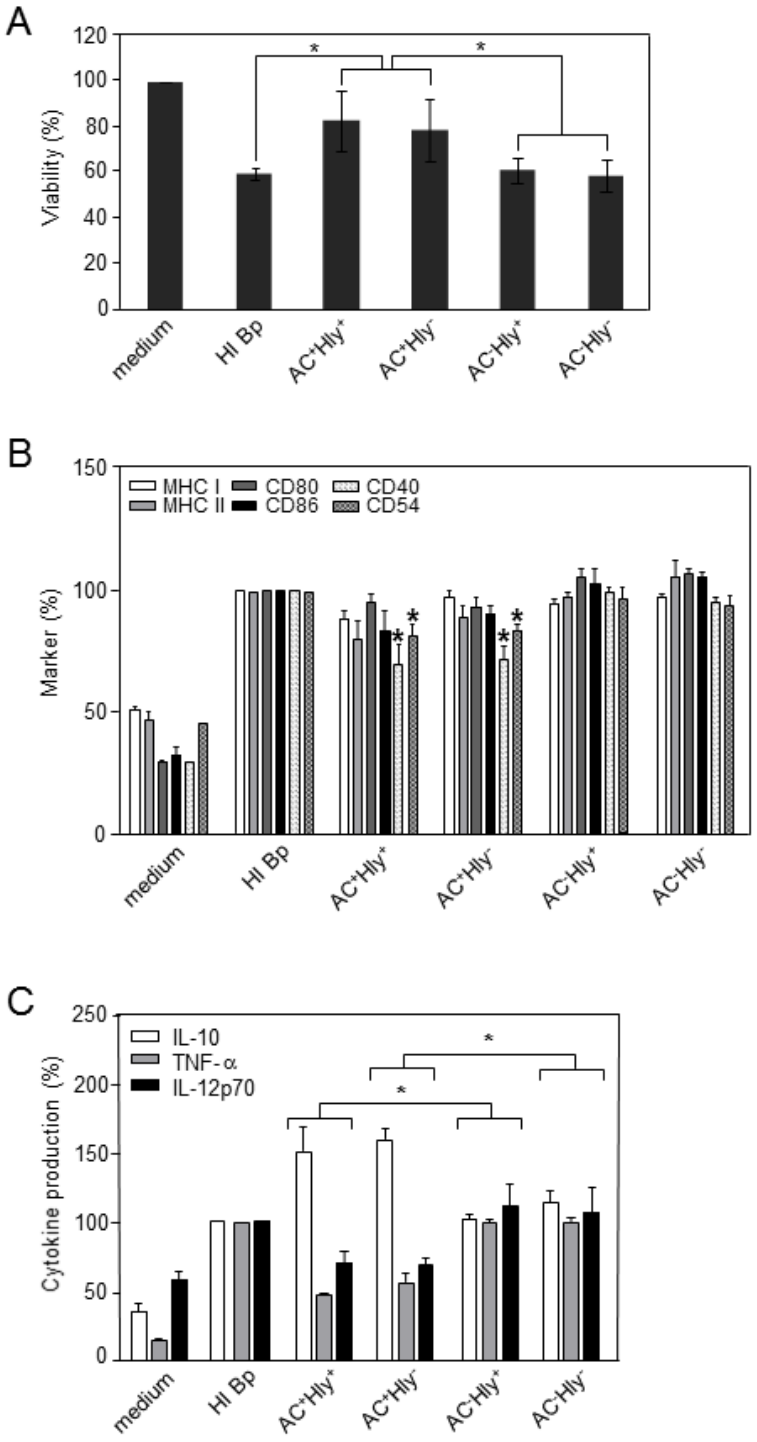
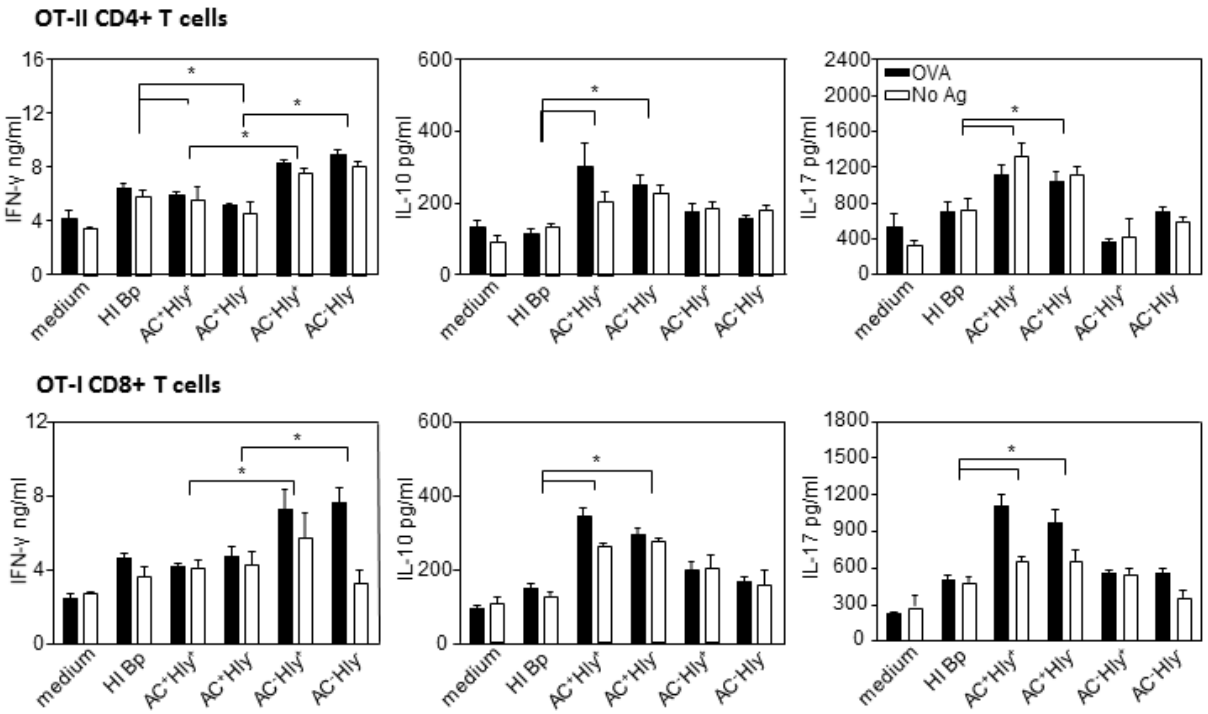


D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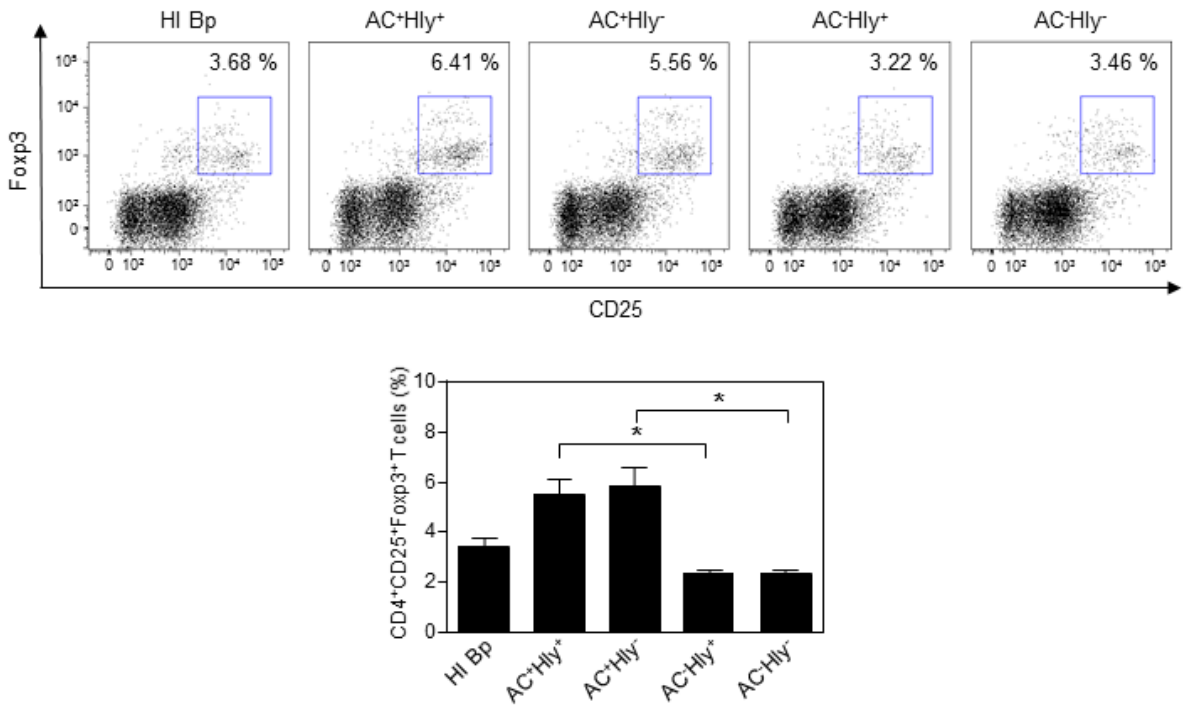
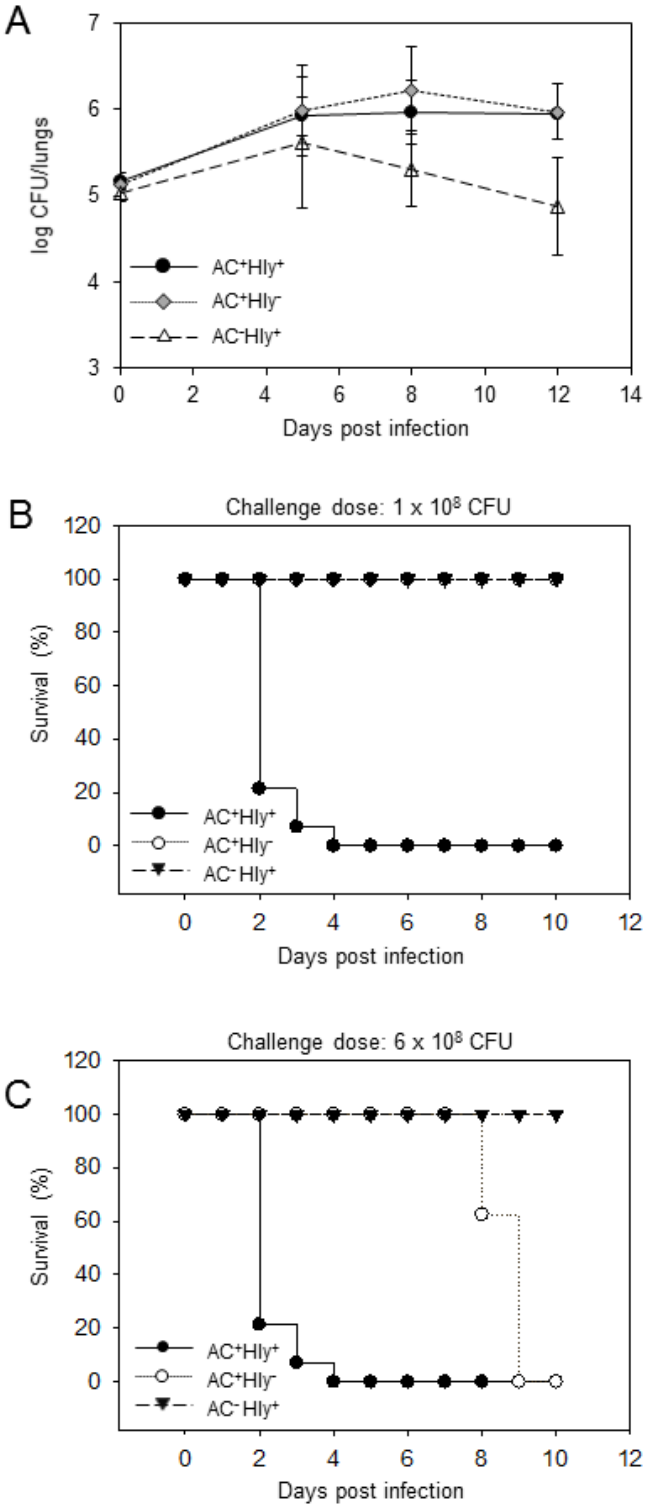
IFN-γ ng/ml
IL-10 pg/ml
IL-17 pg/ml
OVA
No Ag
OT-I CD8+ T cells
medium
HI Bp
AC+Hly+
AC+Hly-
AC-Hly+
AC-Hly-

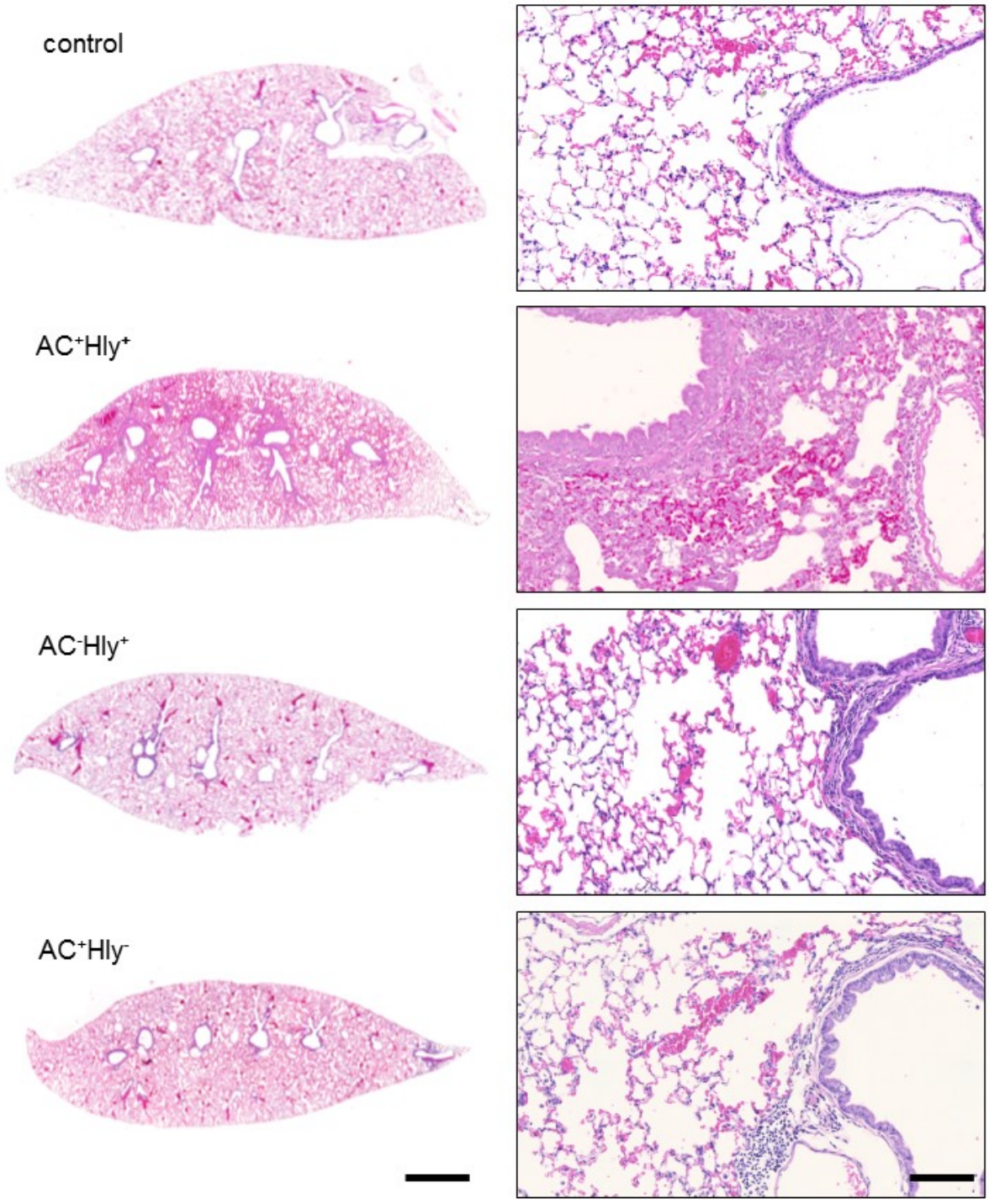
E


HI Bp
AC+Hly+
AC+Hly-
AC-Hly+
AC-Hly-
3.68 %
6.41 %
5.56 %
3.22 %
3.46 %
Foxp3
CD25
CD4+CD25+Foxp3+T cells (%)

Skopova *et al*. Figure 3



Skopova *et al*. Figure 4A



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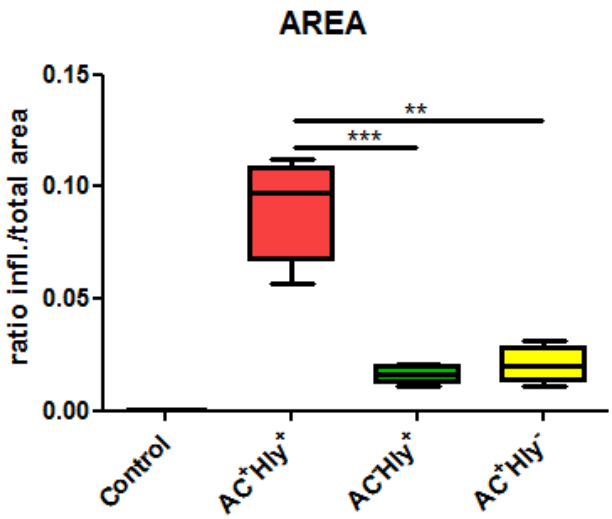
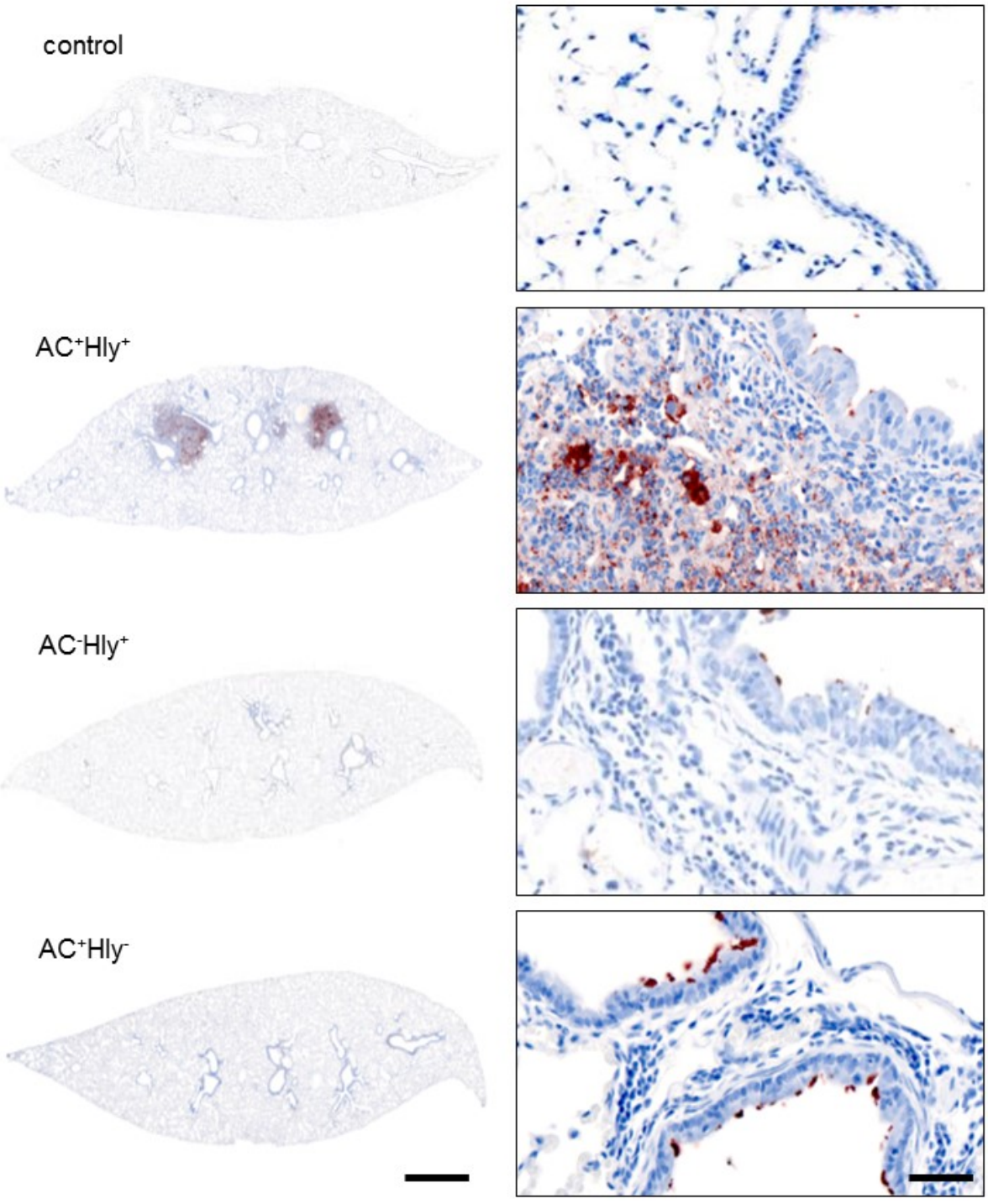


Skopova *et al*. Figure 5



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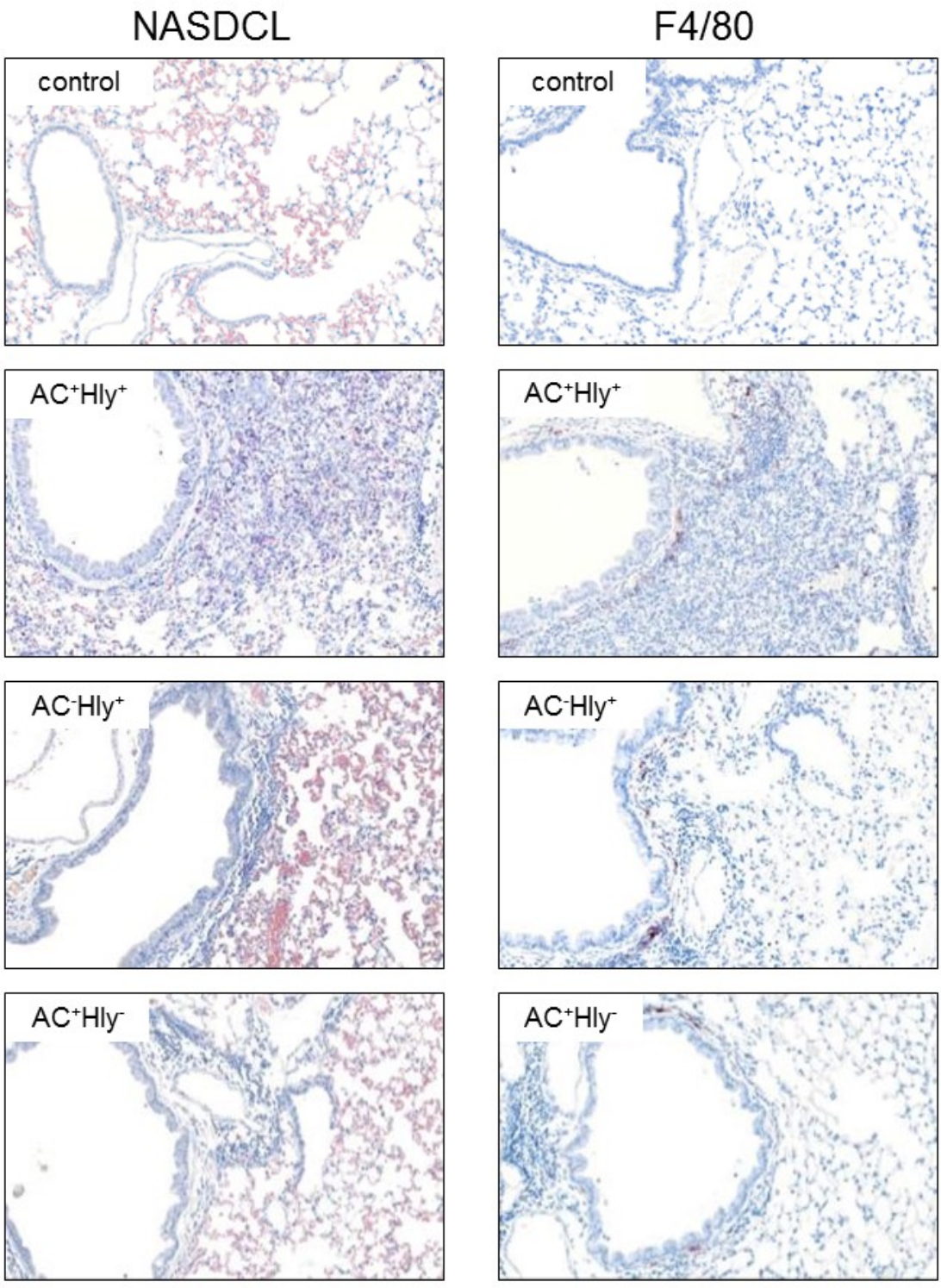

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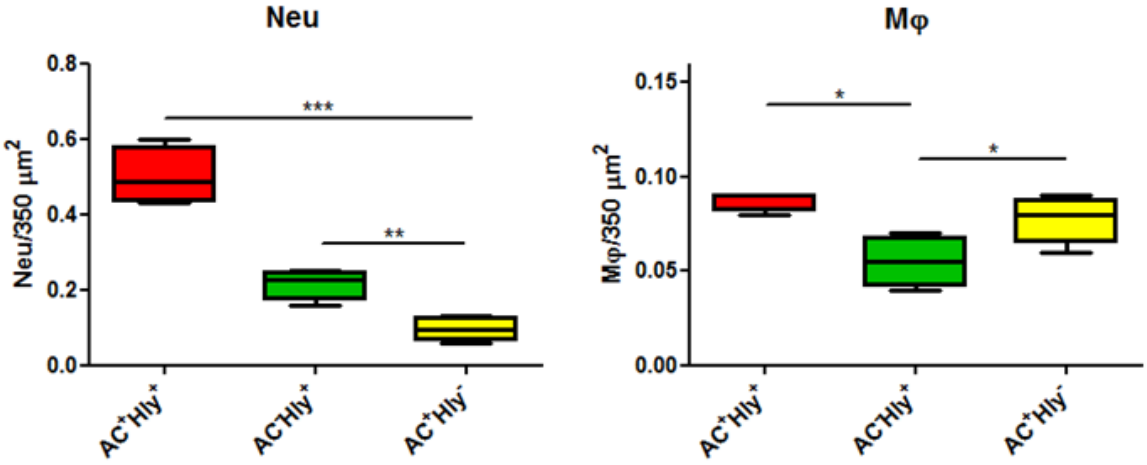

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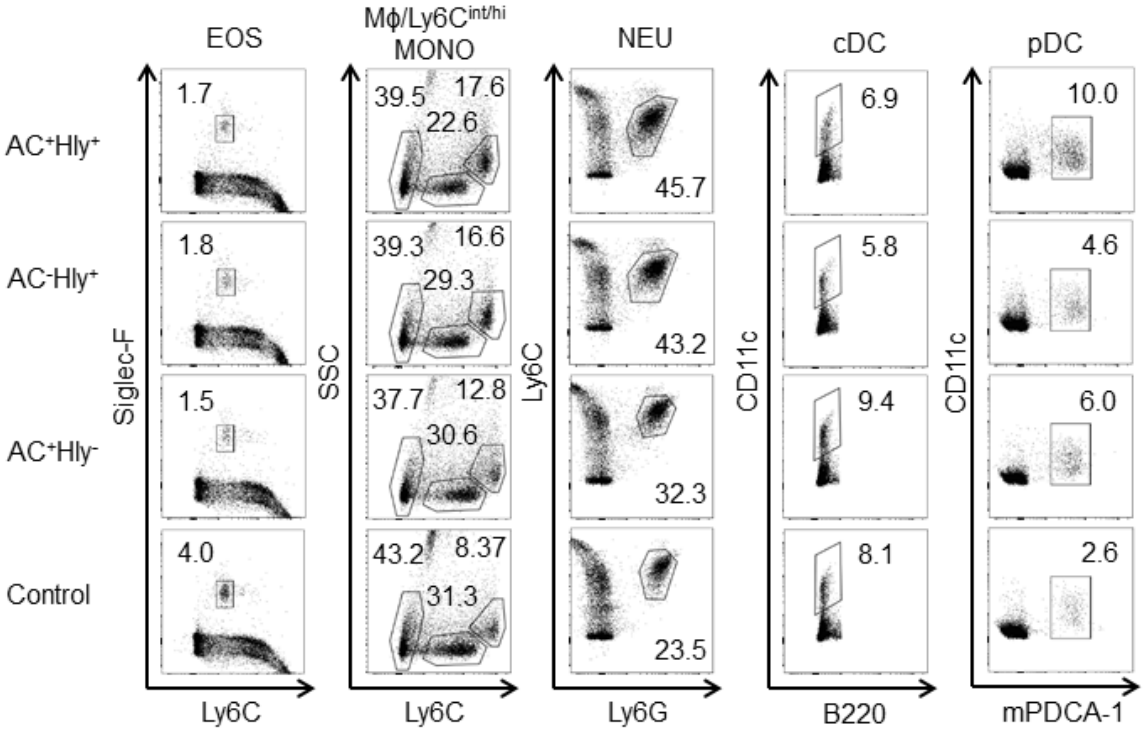

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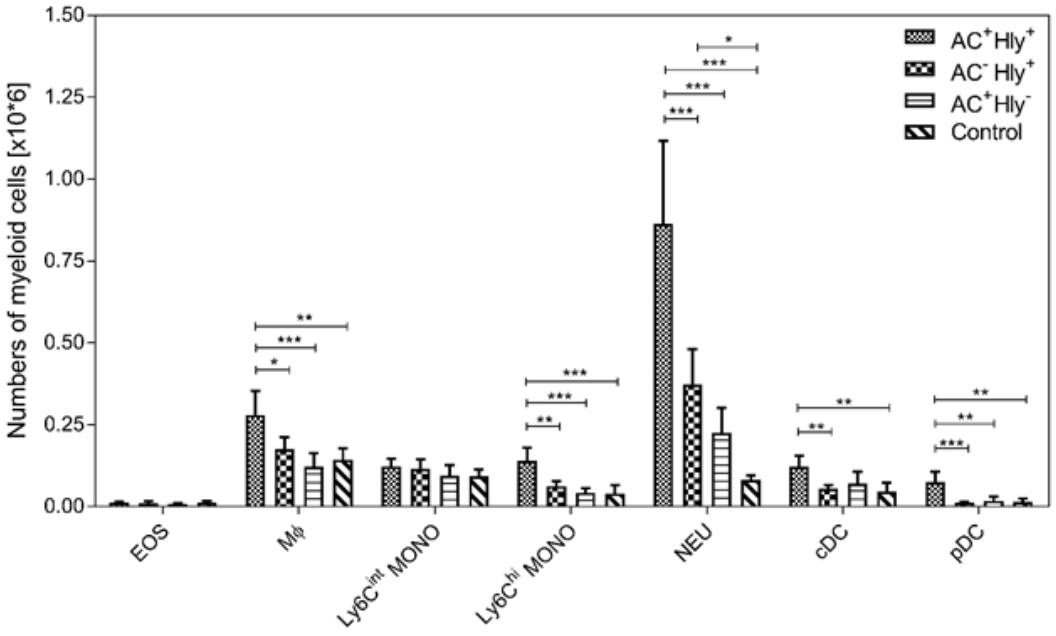
D



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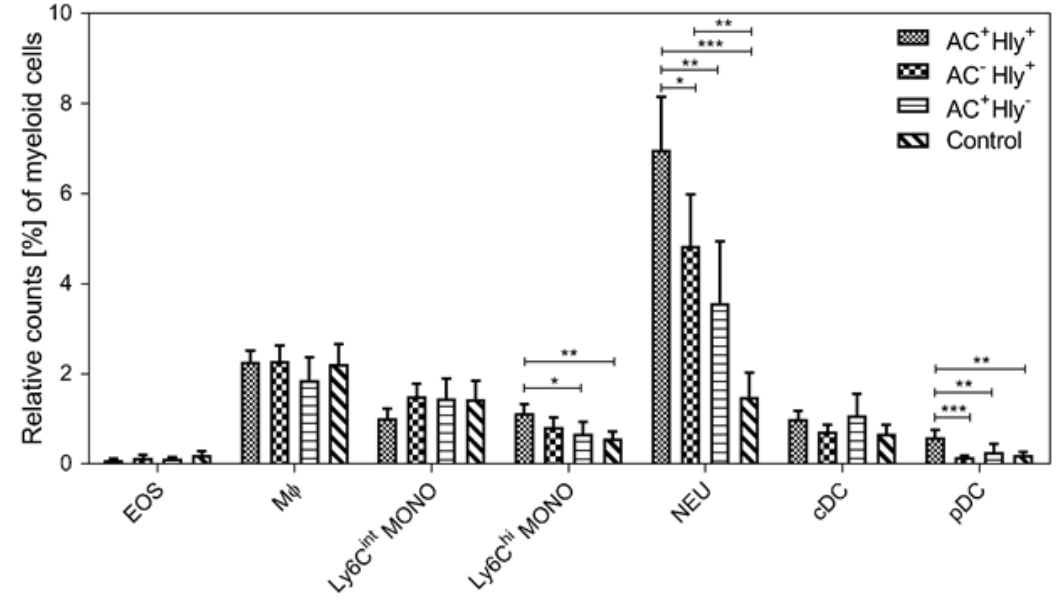
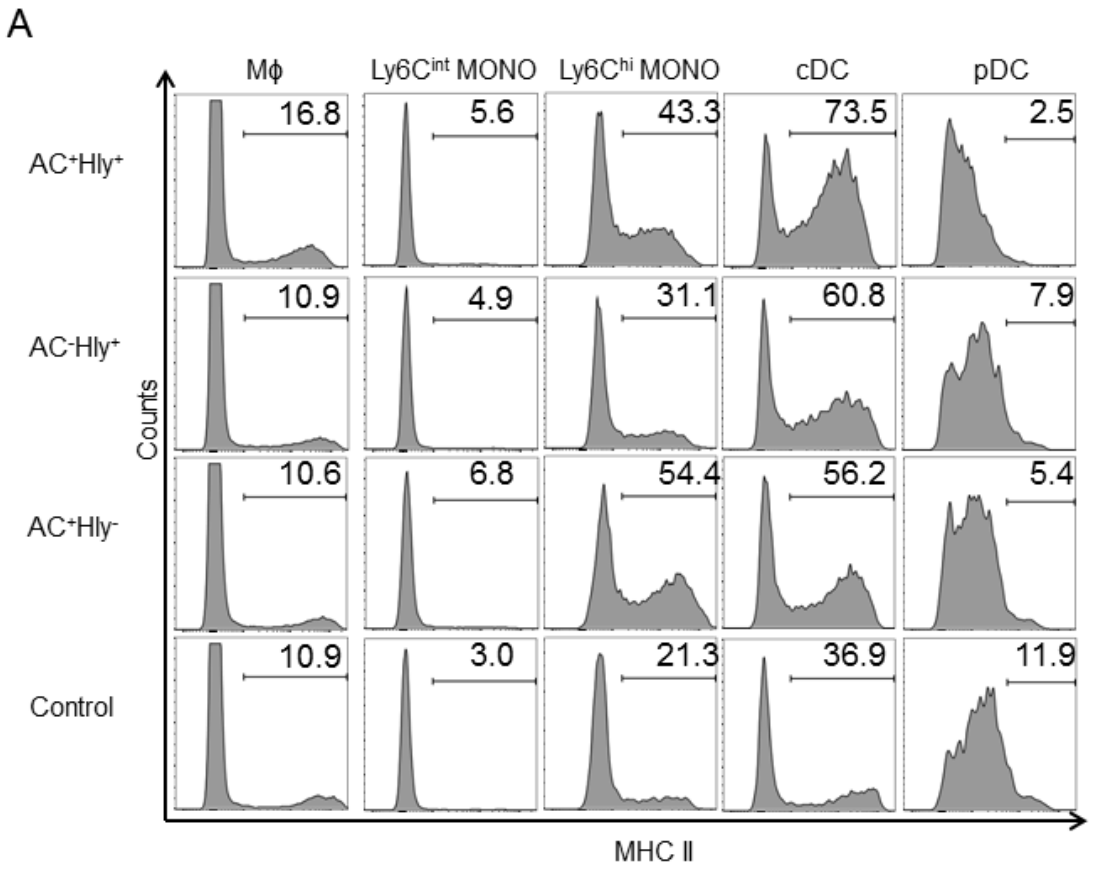
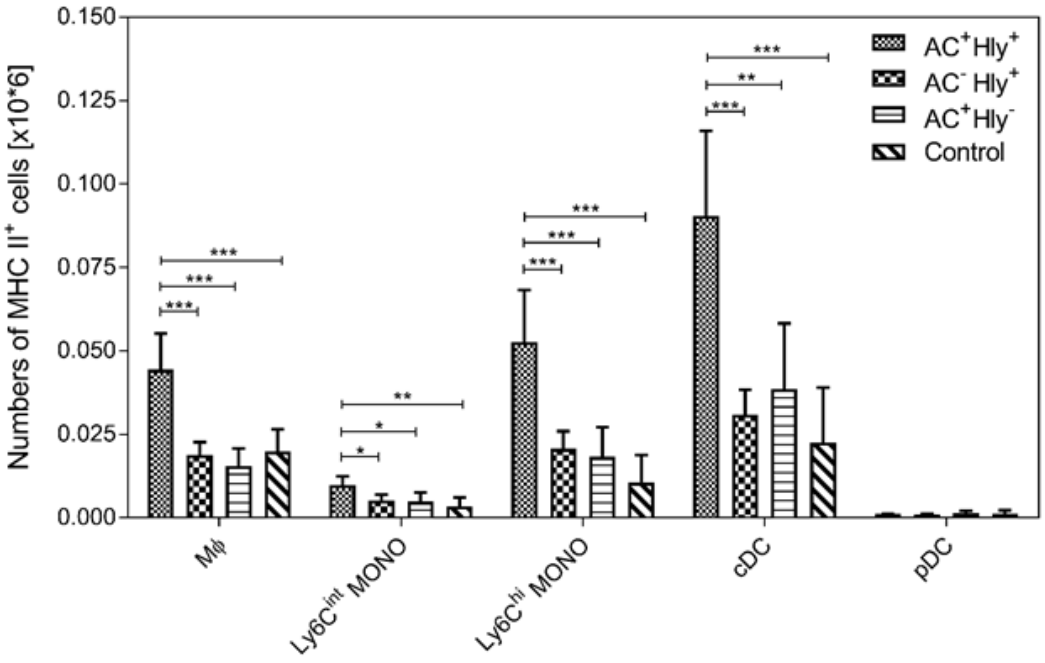


Skopova *et al*. Figure 7A

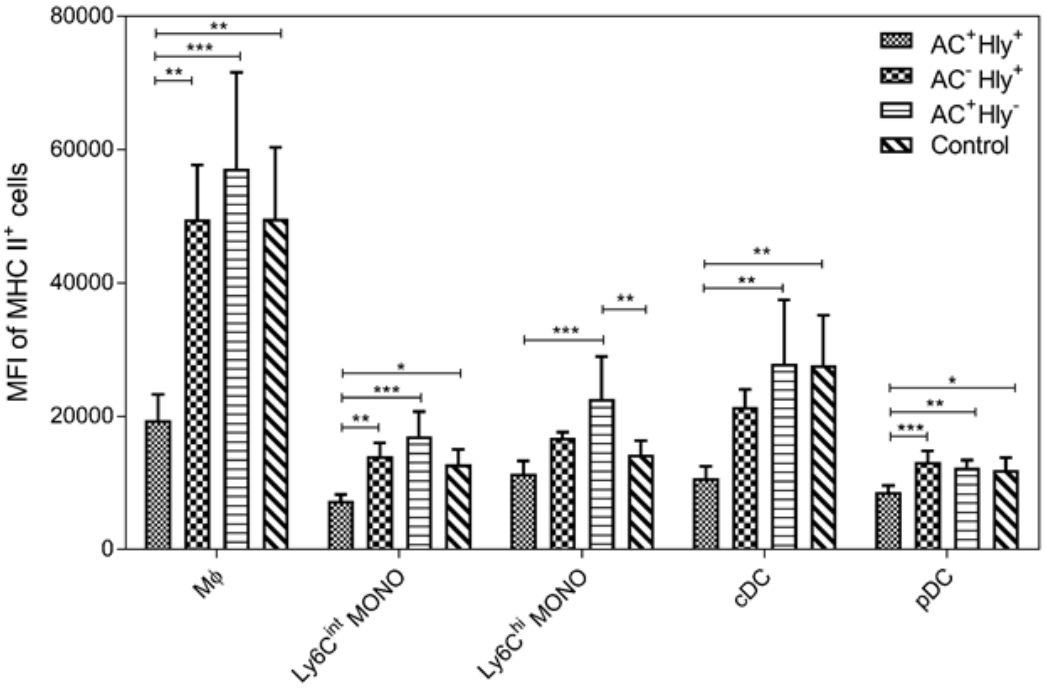


Skopova *et al*. Figure 7B,C

B



C



6.7 PUBLICATION 6

Adkins, I., Holubova, J., **Kosova, M.,** & Sadilkova, L. (2012). Bacteria and their toxins tamed for immunotherapy. *Curr Pharm Biotechnol, 13*(8), 1446-1473.

Bacterial toxins are important virulence factors endowed by specific activities which enable them to manipulate the host immune responses. As useful molecular biology tools, they helped to discover various cellular mechanisms. Moreover, due to their abilities to enter host cells, bacterial toxins or their less toxic mutant variants have been explored for possibility to be used in the field of medicine: to deliver antigens and stimulate the adaptive T cell immune response, to stimulate the immunity as adjuvants, or to eliminate cancer cells. Bacterial toxins usually enter host cells via receptor-mediated endocytosis, except from *Bordetella pertussis* adenylate cyclase toxin, which translocates its adenylate cyclase domain directly across the cellular membrane. *Bordetella pertussis* adenylate cyclase toxin, *Bacillus anthracis* lethal and edema toxins, *Shigella dysenteriae* shiga toxin and *Escherichia coli* shiga-like toxin are good examples of toxins which were reported to have the capacity to transport antigens into host dendritic cells for stimulation of specific T cell responses. *B. pertussis* pertussis toxin, *Vibrio cholera* cholera toxin, *E. coli* heat-labile enterotoxin, or the Cry1A protein of *Bacillus thuringiensis* have the potential to act as adjuvants and stimulate mucosal as well as systemic immune responses. *Corynebacterium diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A-based immunotoxins appear to be very promising tools in cancer immunotherapy, moreover, the DT immunotoxin (DAB(389)IL-2) is already used for treatment of cutaneous T cell lymphoma. Furthermore, currently there are several clinical trials to evaluate the safety and the effectiveness of bacterial toxins derived vaccines, such as *Bordetella adenylate* cyclase toxoid used as a vaccine delivery tool for immunotherapy of cervical tumors and metastatic melanoma.

My contribution: I wrote some parts of manuscript, helped with manuscript finalization.

Bacteria and their Toxins Tamed for Immunotherapy

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Abstract: Bacterial toxins share the ability to enter host cells to target various intracellular proteins and to modulate host immune responses. Over the last 20 years, toxins and their mutated variants, as well as live attenuated bacteria, have been exploited for vaccination and immunotherapy of various infectious, malignant and autoimmune diseases. The ability of *Bordetella pertussis* adenylate cyclase toxin to translocate its adenylate cyclase domain across the host cell membrane, as well as the pathways of intracellular trafficking of *Bacillus anthracis* lethal and edema toxins, *Shigella dysenteriae* shiga toxin or *Escherichia coli* shiga-like toxin, have been repeatedly exploited for the delivery of antigenic epitopes into host cells and for stimulation of antigen-specific T cell responses. Similarly, *E. coli* α-hemolysin, or effector proteins of *Yersinia* and *Salmonella* secreted by the type III secretion systems, were used to facilitate the delivery of fused heterologous proteins or peptides for antigenic presentation. *Vibrio cholerae* cholera toxin, *E. coli* heat-labile enterotoxin, *B. pertussis* pertussis toxin or the Cry1A protein of *Bacillus thuringiensis* have shown a great potential to act as adjuvants and to stimulate mucosal as well as systemic immune responses. The immunotherapeutic potential of some toxins, like *Clostridium perfringens* perfringolysin O, *Streptococcus intermedius* intermedilysin, or *Streptococcus pneumoniae* pneumolysin needs to be evaluated further. The *Bordetella* adenylate cyclase toxoid used as a vaccine delivery tool, or *Corynebacterium diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A-based immunotoxins, are currently in various phases of clinical trials for cancer immunotherapy, as are some antigen-delivering *Salmonella* and *Listeria monocytogenes* strains.

Keywords: Adjuvant, antigen delivery, antigen presentation, bacteria, cancer immunotherapy, toxin, vaccination.

INTRODUCTION

The successful stimulation of T cell adaptive immune responses crucial for the elimination of infection or cancer cells lies within the capacity of antigen presenting cells (APCs), such as dendritic cells (DC) or macrophages, to process and present antigens on MHC class I and II molecules to T cells. Exogenous antigens internalized by endocytosis are processed within the endosomal system of APC into peptides that are loaded on MHC class II molecules and transported to the cell surface where they can be recognised by antigen-specific $CD4^+$ T cells. Antigens which are present or have gained access to the host cell cytosol via cross-presentation are processed mostly by proteasome into peptides and transported to the endoplasmic reticulum where they are loaded on MHC class I molecules. On the cell surface, MHC class I-peptide complexes are recognized by $CD8^+$ cytotoxic T cells (CTLs) which play a crucial role in the elimination of viruses and intracellular bacteria as well as in the eradication of tumors. However, many pathogens have evolved sophisticated strategies to elude antigen processing and the presentation machinery of APCs, thereby ensuring survival within the host cells [1]. Similarly, tumor cells display characteristics which suppress their recognition and elimination from the organism [2]. Therefore there is a great need of vectors that are capable of delivering specific pathogen-related or tumor-associated antigens into APCs as well as for immunological adjuvants which would boost inefficient host immune responses. Several bacterial toxins and their non-toxic mutants have been intensively studied over the past 20 years in order to harness their abilities to enter host cells for the stimulation of T cell adaptive responses, direct elimination of cancer cells or to boost immunity as adjuvants. Moreover, truncated *Escherichia coli* α-hemolysin or effector proteins of the type III secretion system of *Yersinia* or *Salmonella* have been used to facilitate the delivery of heterologous antigens into APCs.

With the exception of *Bordetella pertussis* adenylate cyclase toxin which is able to translocate its enzymatic adenylate cyclase domain directly across the cellular membrane, most toxins enter host cells predominantly via receptor-mediated endocytosis and are relocated through endosomal system or a retrograde transport involving Golgi apparatus and endoplasmic reticulum into the host cell cytosol where they modulate various cellular proteins. *B. pertussis* adenylate cyclase toxin and pertussis toxin, *Vibrio cholerae* cholera toxin, *E. coli* heat-labile enterotoxin and *Bacillus anthracis* edema toxin induce the elevation of cAMP, a key second messenger, in the host cells which affects various signal transduction pathways [3]. *Shigella dysenteriae* shiga toxin, *E. coli* shiga-like toxins, *Corynebacterium diphtheriae* diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A attack components of protein synthesis machinery, thereby inducing apoptosis. Similarly, *Bacillus anthracis* lethal factor induces apoptosis of host cells by cleaving mitogen-activated protein kinase kinases [4]. Other toxins such as

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Listeria monocytogenes listeriolysin O, *Clostridium perfringens* perfringolysin O, *Streptococcus intermedius* intermedilysin, pneumolysin of *Streptococcus pneumoniae* or crystal (Cry) proteins of *Bacillus thuringiensis* cause cell lysis by forming pores in the host cell membrane. The toxic activities of bacterial protein toxins as well as pathogenic bacteria exploited as live carriers for vaccination can be genetically manipulated for our benefit. Therefore the main objective of this review is to summarize the knowledge on their use as antigen delivery tools, adjuvants for co-administered antigens, and importantly, as cytotoxins and immunotoxins for immunotherapy of cancer. The impact of these findings on future therapies and disease management will be briefly discussed. Some other toxins such as botulinum neurotoxin of *Clostridium botulinum* which is used in the treatment of various neurological and muscle tone disorders or in the cosmetic industry, cytotoxic necrotizing factor 1 (CNF1) of *E. coli* which has been reported to improve learning and memory [5] and to exhibit adjuvant properties [6] as well as zonula occludens toxin (ZOT) of *V. cholerae* which showed a potential to be used as a drug delivery agent [7] and vaccine adjuvant [8] are not discussed herein as their use in immunotherapy have been extensively reviewed elsewhere [9].

ADENYLATE CYCLASE TOXIN OF *BORDETELLA PERTUSSIS*

Adenylate cyclase toxin (CyaA) is an important virulence factor of *B. pertussis*, the causative agent of whooping cough as it has been shown that CyaA-deficient bacteria were unable to cause lethal infection and were cleared rapidly from the lungs of mice [10]. CyaA is a 1706 amino acids long protein molecule composed of N-terminal adenylate cyclase (AC) domain (~ 400 residues) which upon binding of calmodulin in the host cell catalyzes unregulated conversion of cellular ATP to cAMP. The translocation of AC domain is connected to the influx of Ca^{2+} ions into the host cells [11]. The C-terminal RTX hemolytic moiety (~ 1306 residues) accounts for the binding to the $\alpha_M\beta_2$ integrin, CD11b/CD18 (CR3, Mac-1) [12] expressed predominantly on host myeloid phagocytes, for translocation of AC domain into the cell cytosol and for making the cell permeable by cation-selective toxin pores, thereby perturbing ion homeostasis [13]. CyaA inserts into cytoplasmic membrane of host cells and translocates its AC domain without the need for endocytosis [14]. Upon *Bordetella* colonization, CyaA interacts with tracheal epithelial cells, which aids the adhesion of bacteria to ciliated mucosa [15] and induces production of IL-6 [16]. In phagocytic cells expressing CD11b/CD18 receptor such as macrophages, DC or neutrophils, CyaA-generated cAMP subverts their phagocytic and macropinocytic capacities, chemotaxis and oxidative burst [17, 18, 19] which eventually leads to the induction of cell death [20, 21]. It has been shown that CyaA alters LPS-induced DC maturation and inhibits the production of proinflammatory cytokines like TNF-α or IL-12p70, whereas it potentiates secretion of IL-6 and IL-10 thereby affecting T cell adaptive responses [22, 23, 24]. CyaA was shown to induce Th17 cells [25] via inflammasome activation [26] and T cells with a regulatory phenotype [23].

Interestingly, Bagley *et al.,* reported that CyaA toxin itself, without other stimuli, induced maturation in human DC and increased their ability to present alloantigens to naïve $CD4^+$ T cells [22] which was dependent on its ability to generate cAMP within the host cells. These results were confirmed by Ross *et al.,* using DC hyporesponsive to LPS isolated from C3H/HeJ mice [23]. Moreover, CyaA was documented to enhance antibody levels to co-administered antigen *in vivo* [23, 27] which suggest a certain adjuvant potential of this toxin. However, data on the adjuvant modulation of immune cell functions by CyaA-induced cAMP signaling as well as on its possible therapeutic value are lacking.

Over the past 15 years, CyaA has mainly been exploited as a non-replicative vector for antigen delivery [28, 29, 30]. Specifically, genetically detoxified CyaA-AC^- (CyaA-E5) toxoids with disrupted ability to catalyze conversion of cytosolic ATP to cAMP have been shown to efficiently deliver antigenic epitopes for presentation in the complex of MHC class I and II molecules of APCs which yielded the induction of antigen specific T cell responses (Table **1**). Genetic ablation of the AC activity does not affect invasiveness, however, the efficiency of epitope delivery seems to be dependent on the position of insertion within the AC domain, the electrostatic charge of the inserted sequence, and length and nature of the inserted epitope [29-33]. Nevertheless, it has been observed that CyaA-AC^- toxoids are able to deliver epitopes with 100 times higher molar efficiency than the recombinant protein alone [34]. Already a single copy of $CD8^+$ T cell-restricted epitope from lymphocytic choriomeningitis virus (LCMV) inserted in a recombinant CyaA was shown to induce specific CTLs as well as to provide protection of mice against lethal challenge with LCMV [28, 35, 36]. Similarly, CyaA-AC^- molecule carrying several $CD8^+$ T cell epitopes induced multiple CTL responses *in vivo* [37]. The induction of specific CTLs occurred without the need for adjuvant or help of $CD4^+$ T cells [35, 38]. CyaA-AC^- toxoids were also shown to deliver $CD4^+$ T cell epitopes into MHC class II presentation pathway inducing specific Th1 type T cell responses *in vivo* [30, 38]. Moreover, CyaA-AC^- carrying epitopes of Tat protein from HIV type 1 virus was found to stimulate B cell reponse and the production of specific neutralizing antibodies [39]. Most importantly, CyaA-AC^- toxoids have been demonstrated to elicit protective and therapeutic immunity against HPV-16-induced tumors and melanoma in mice [40-43]. The therapeutic efficacy could be enhanced by heterologous booster immunization and the previous immunity towards CyaA seems to have little effect on immune responses [42, 43]. Since also human DC were shown to process and present CyaA-AC^--delivered melanoma epitopes to human CTL clones [41], preparation of phaseI/II clinical trials of vaccines for immunotherapy of cervical tumors [44], and metastatic melanoma are underway [45]. CyaA-AC^- was further proven to be a useful diagnostic tool as toxoids bearing ESAT-6 or CFP-10 proteins of *M. tuberculosis* were used in recall response assays for diagnosis of latent tuberculosis [46]. Vaccination with CyaA-AC^- carrying mycobacterial antigens failed to confer protective immunity against *M. tuberculosis* [31, 34], while prime-boost immunization with CyaA-AC^- carrying an epitope from circumsporozoite protein of *Plasmodium berghei* epitope together with anti-CTLA 4 antibodies allowed to elicit protection against liver-stage malaria in mice [47, 48].

Table 1. Adenylate Cyclase Toxin (CyaA) of *B. pertussis* as a Vector for Antigen Delivery

Antigen	Origin	Carrier	Immune Response (Model; CMR)[a]	Protection in Animal Model[b]	Ref.
$NP_{118-132}$	Nucleoprotein of lymphocytic choriomeningitis virus	CyaA wt[c]	*In vitro* CTL-Mediated target cell lysis	ND	[28]
	(LCMV)	CyaA wt CyaA-AC$^-$	BALB/c; CTL	ND	[35]
		CyaA-wt CyaA-AC$^-$	BALB/c; CTL	+	[36]
		CyaA-AC$^-$	BALB/c; CTL; $CD4^+$ T cells (Th1)	ND	[38]
		CyaA-AC$^-_{cys}$	BMDC, splenocytes, C57BL/6, BALB/c; CTL	ND	[54]
		CyaA-AC$^-$	BALB/c; CTL Multiepitope containing also $OVA_{257-264}$ and $V3_{316-327}$	+ (LCMV)	[37]
$OVA_{257-264}$	Chicken egg ovalbumin	CyaA-AC$^-$	C57BL/6; CTL	+	[40]
		CyaA-AC$^-$	$CD8^+$ T cell hybridoma (B3Z)	ND	[29]
		CyaA-AC$^-_{cys}$	BMDC, splenocytes, C57BL/6, BALB/c; CTL	ND	[54]
$MalE_{126-140}$	Maltose-binding protein of *E. coli*	CyaA-AC$^-$	C57BL/6, BALB/c; $CD4^+$ T cell hybridomas (CRMC3 and FBCD1)	ND	[30]
$V3_{316-327}$	HIV	CyaA wt CyaA-AC$^-$	BALB/c; CTL	ND	[35]
Tat		CyaA-AC$^-$	BALB/c, CTL, $CD4^+$ T cells (Th1); neutralizing Ab	ND	[39]
$Tyr_{369-377}$ GnT-V	Melanoma epitopes: Tyrosinase (Tyr) N-acetylglucosaminyl-transferase V (GnT-V)	CyaA-AC$^-$	HHD mice expressing HLA*0201; human DC; CTL	ND	[41]
E7, $E7_{49-57}$ $E7_{\Delta 30-42}$	Human papillomavirus 16 (HPV16)	CyaA-AC$^-$	C57BL/6; CTL, $CD4^+$ T cells (Th1)	+	[42]
		CyaA-AC$^-$	BMDC, C57BL/6	+	[43]
ESAT-6 CFP10	*M. Tuberculosis*	CyaA-AC$^-$	IFN-γ production by bovine PBMC; CFP10-specific $CD4^+$T cell line	ND	[46]
		CyaA-AC$^-$	IFN-γ production by human PBMC; $CD8^+$ and $CD4^+$ T cells	ND	[55]
Ag85A		CyaA-AC$^-$	BMDC, BALB/c, C57BL/6; $CD4^+$ T cells (Th1)	-	[34]
TB10.4 $TB10.4_{74-88}$ $TB10.4.4_{20-28}$ $TB10.4.4_{15-33}$		CyaA-AC$^-$	BMDC, BALB/c; CTL, $CD4^+$ T cells	-	[33]
$CSP_{245-253}$	*P. Berghei*	CyaA-AC$^-$	BALB/c; CTL	+/-	[47]
		CyaA-AC$^-$	BALB/c; CTL	+	[48]

[a] Abbreviations: CMR, cell-mediated response; CTL, cytotoxic T lymphocytes; Ab, antibody response; Cys, cysteine; BMDC, bone marrow-derived dendritic cells; PBMC, peripheral blood mononuclear cells.

[b] +, 100 % protection; -, no protection; +/-, partial protection; ND, not determined.

[c] CyaA wt, wild type enzymatically active CyaA toxin; CyaA-AC$^-$, CyaA toxoid with ablated adenylate cyclase activity.

Detoxified CyaA-AC$^-$ was also shown to enhance antibody response to co-administered antigens [49, 50]. Such adjuvanticity, together with its Th1 polarizing activity [37], might be dependent on its ability to induce Ca^{2+} influx and K^+ efflux, which are not abolished by the detoxification, in host cells [11, 51]. Ca^{2+} signalling was shown to stimulate DC maturation [52]. Similarly, K^+ ions have been shown to activate inflammasome and IL-1β production in LPS-stimulated macrophages and DC [26, 53]. However, it remains to be determined if CyaA-AC$^-$ toxoid acts as an adjuvant on immune cells via signaling mediated by these ions.

ANTHRAX TOXINS OF *BACILLUS ANTHRACIS*

The spore-forming, Gram-positive *B. anthracis* is the causative agent of anthrax in animals and humans. The anthrax toxins consist of a protective antigen (PA; 83 kDa), lethal factor (LF; 90 kDa) and edema factor (EF; 89 kDa) which individually are non-toxic. The combination of PA and LF, called lethal toxin causes death of experimental animals [56], whereas the combination of PA and EF, known as edema toxin, induces an increase in intracellular cAMP levels in susceptible cells [57] and elicits skin edema after subcutaneous injection [58]. PA binds to the cell-surface receptors tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, expressed as different isoforms by many cell types, including immune cells [59, 60]. The cleavage of the receptor-bound PA by cell surface proteases, such as furin, results in the release of the N-terminal 20 kDa fragment [61]. The remaining 63 kDa fragment of PA then oligomerizes to form ring-shaped heptamers which competitively bind LF or EF [62, 63], and this entire complex undergoes receptor-mediated endocytosis. The low pH of the endosome causes major conformational changes in the PA molecule, leading to the insertion into the endosomal membrane [64]. LF and EF are subsequently translocated across the endosomal membrane into the cell cytosol [65] where they exert their toxic effects. While LF is a zinc-dependent metalloproteinase that cleaves most isoforms of mitogen-activated protein kinase kinases within mammalian cells [4], EF is a calmodulin-dependent adenylate cyclase that causes an increase in intracellular cAMP levels of host cells [57]. Both LT and ET were shown to incapacitate bactericidal functions of immune cells by inhibiting phagocytosis, oxidative burst, cell motility and inducing apoptosis [66, 67]. LT was shown to inhibit DC maturation and secretion of pro-inflammatory cytokines, thereby affecting T cell adaptive responses [68]. LT and ET can also directly inhibit T cell [69] and B cell activation [70].

Lethal factor is a 796 amino acids long polypeptide. The N-terminal truncated LF (1-255 amino acids) contains the information necessary for PA binding [71, 72] and lacks any toxic activity [73] which has been exploited for delivering antigens into APCs (Table **2**). Successful CD8$^+$ T cell induction has been demonstrated after the injection of PA + LFn carrying genetically fused antigenic epitope from listeriolysin O of *L. monocytogenes* ($LLO_{91\text{-}99}$) or chemically coupled epitope from chicken egg ovalbumin ($OVA_{257\text{-}264}$) [74, 75] in mice. The immune response was stimulated by as little as 30 fmol of the fusion protein [75]. Similarly, large polypeptides and protein antigens may be introduced by PA + LFn delivery system to induce specific CTLs [76-79] and CD4$^+$ T cells [80, 81] which leads to the generation of memory T cells [77, 82]. Mice immunized with PA + LFn-$LLO_{91\text{-}99}$ or LFn-$NP_{118\text{-}126}$ or a multiepitopic LFn-$NP_{118\text{-}126}$-$LLO_{91\text{-}99}$ were protected against lethal *L. monocytogenes* or LCMV challenge, respectively [74, 77]. Interestingly, CD4 knockout mice were unable to generate a CTL response when treated with PA + LFn-epitope fusion proteins, suggesting that CD4$^+$ T helper cells responses are essential [83]. Even though PA itself does not seem to possess any adjuvant activity [74], it can generate an antibody response that might prevent intoxication of animals after exposure to *B. anthracis*. Ballard *et al.,* showed that immunization with a small amount of protein neither generated any detectable antibody response nor prevented the use of this system for subsequent vaccination [83]. Dolling *et al.,* observed that a long LFn-$NP_{118\text{-}404}$ construct can prime a CTL response in the absence of PA [77]. Later it has indeed been shown that a high dose of LFn can deliver antigens and stimulate CTLs without the need of PA [80, 84].

The proteolytic activation of PA has been exploited for cancer immunotherapy to generate tumor cell-selective cytotoxins. Mutated PA proteins, in which the furin cleavage site was replaced by sequences cleaved specifically by cancer specific urokinase plasminogen activator, were constructed [85]. These mutated proteins in combination with a recombinant LT fused with *P. aeruginosa* exotoxin A (FP59) [71] were activated selectively on the surface of tumor cells and exhibited potent tumoricidal activity *in vitro* [85] and *in vivo* [86]. In addition, the property of PA to bind TEM8, which is over-expressed during tumor angiogenesis [87], has been exploited to generate mutated PA variants with a higher receptor affinity [88]. LT exhibits a broad anti-tumor efficacy via targeting of tumor angiogenesis [89, 90]. However, to limit the toxicity of LT to endothelial cells, mutated PA (PA-L1) was created carrying a specific cleavage site for matrix metalloproteinases which are specifically upregulated in angiogenic lesions [91]. Initial *in vivo* studies revealed that the treatment with PA-L1 + LF induced 90 % tumor growth inhibition and 30 % complete regressions of the V600E B-RAF melanoma cell line C32 xenografts [92]. Additionally, PA can be used for imaging of cells expressing a specific cell-surface proteolytic activity [93] or a vehicle for the delivery of antisense *PNA* oligomers into cells [94].

Edema factor has also been exploited as a potential antigen delivery tool as it has been found that N-terminal domain of EF (EFn; 1-260 residues) lacks cytotoxicity and binds fully to PA [63]. Chandra *et al.,* showed that PA + EFn carrying ESAT-6 protein of *M. tuberculosis* elicited CTLs and induced Th1 and Th2 cytokines *in vitro* [95]. The enzymatically active ET was shown to act as an adjuvant for co-administered antigens and to induce OVA- and PA-specific CD4$^+$ T cells which secreted IFN-γ and selected Th2 cytokines in mice [96]. Similarly to other bacterial toxins with cAMP-inducing potential ET was reported to stimulate maturation and migration of DC [96, 97].

Table 2. Anthrax Toxins of *B. anthracis* as Vectors for Antigen Delivery

Antigen	Origin	Carrier	Immune Response (Model; CMR)[a]	Protection in Animal Model[b]	Ref.
LLO_{91-99}	Listeriolysin O of	PA + LFn[c]	BALB/c; CTL	+/-	[74]
	L. monocytogenes	PA + LFn	BALB/c; CTL	ND	[83]
		PA + LFn	BALB/c, C57BL/6; CTL	ND	[77]
$OVA_{257-264}$	Chicken egg ovalbumin	PA + LFn_{cys}	C57BL/6; CTL	ND	[75]
$OVA_{323-339}$		PA + LFn	BALB/c; CTL	ND	[83]
		PA + LFn, LFn	BMDC, C57BL/6; CTL, $CD4^+$ T cells	ND	[81]
$NP_{118-126}$	Nucleoprotein of lymphocytic choriomeningitis virus	PA + LFn	BALB/c; CTL	ND	[83]
	(LCMV)	PA + LFn	BALB/c, C57BL/6; CTL	+	[77]
		PA + LFn	BALB/c; CTL, Memory T cells	ND	[82]
ESAT-6	*M. Tuberculosis*	PA + LFn	BALB/c; CTL	ND	[79]
		PA + EFn[d]	BALB/c; CTL,$CD4^+$ T cells	ND	[95]
gp120	HIV	PA + LFn	BALB/c; CTL	ND	[76]
V3, gag p24,		PA + LFn, LFn	Human PBMC, rabbit, BALB/c; CTL; $CD4^+$ T cells	ND	[78, 80]
nef		LFn	BALB/c; CTL; + alum	ND	[84, 98]

[a] Abbreviations: CMR, cell-mediated response; CTL, cytotoxic T lymphocytes; Cys, cysteine; BMDC, bone marrow-derived. Dendritic cells; PBMC, peripheral blood mononuclear cells.

[b] +, 100 % protection; +/-, partial protection; ND, not determined.

[c] LFn, N-terminal 254-255 amino acids of lethal factor; PA, protective antigen.

[d] EFn, N-terminal 260 amino acids of edema factor.

CHOLERA TOXIN OF *VIBRIO CHOLERAE* AND HEAT-LABILE ENTEROTOXIN OF *ESCHERICHIA COLI*

Cholera toxin (Ctx) of *V. cholerae* and related heat-labile enterotoxin (Etx) produced by enterotoxigenic strains of *E. coli* (ETEC) have been known for many years as causative agents of cholera and travellers' watery diarrhoea, respectively. Recent studies have revealed that Ctx and Etx enhance enteric bacterial colonization and pathogenicity [99, 100]. Both toxins form oligomers (AB5) composed of a single A subunit (CtxA or EtxA), that contains two domains (A1 and A2) linked together by a loop formed by a disulphide bridge, and five identical B subunits (CtxB or EtxB) sharing almost 80 % identity in nucleotide sequence which are highly resistant to proteolytic degradation and low pH [101]. Upon infection Ctx is secreted across the bacterial outer membrane into the extracellular environment [102], while Etx is probably released after permeabilisation of bacteria by host bile salts and proteinases [103] or via the outer membrane vesicles produced by ETEC [104]. Once released, both toxins bind with extremely high affinity to the host cell-surface GM1 ganglioside receptors via their B subunits [105]. Moreover, Etx displays a more promiscuous receptor-binding activity interacting also with asialo-GM1, lactosylceramide and certain galactoproteins [106, 107]. The interaction of B subunits with the receptor is crucial for uptake, internalisation and intoxication of target cells [108]. After internalisation, the A subunit is activated by a proteolytic cleavage and toxins are delivered via Golgi apparatus into the endoplasmic reticulum using a retrograde pathway. The A subunit possessing an ADP ribosyltransferase activity is translocated into the cytoplasm where it acts on trimeric G proteins which results in the activation of adenylate cyclase and the increase of intracellular cAMP [109]. This leads to chloride and water secretion from intestinal cells, and diarrhoea [110].

Ctx and Etx have long been known as effective mucosal immunogens. Both toxins are able to stimulate strong mucosal immunity either to themselves and to co-administered antigens [111, 112]. This adjuvant activity makes them interesting candidates for vaccine development. Unfortunately, their toxicity complicates their use as components of human vaccines. To avoid toxic effects, Lycke *et al.,* constructed mutant Ctx/Etx proteins lacking the ADP-ribosylating activity, however, no adjuvant effect upon oral administration could be detected [113]. Nevertheless, the ADP-ribosylating activity does not seem to be the only feature of both toxins responsible for their adjuvant properties as later Giuliani *et al.,* showed that a single point mutation in the A subunit of Etx (LTR72) lowered the toxicity of the protein to 0.6 % compared to the wild type Etx, and still retained its adjuvant

potential [114]. Moreover, when LTR72 was administered intranasally, even higher mucosal response to co-administered antigen in comparison to the wild type was detected. The research on adjuvant properties of B subunits, CtxB/EtxB, revealed that they are entirely dependent on their receptor binding activity [115-117] and that EtxB exhibits a more potent adjuvant than CtxB [118]. All together, the enzymatic activity and structural properties of the A subunit [119] together with the G_{M1}-binding activity of the B subunit [116, 117], all contribute to the adjuvanticity of Ctx both toxins [110, 120].

CtxB subunit has been tested as a part of cholera vaccine in clinical trials [121]. Recombinant CtxB carrying epitopes from HIV gp120 or serin-rich *Entamoeba histolytica* protein induced an antibody response in mice [122, 123]. EtxB has also been shown to deliver an antigenic epitope from hepatitis B surface antigen into the cells and to induce an antibody response *in vivo* [124]. In addition to this, oral microencapsulated anti-colonization factor 6 antigen (meCS6) vaccine containing EtxB with mutation R192G (LT-R192G) as adjuvant, against enterotoxigenic *E. coli* showed some success in clinical trials on safety and immunogenicity [125].

The fact that the main receptor G_{M1} ganglioside is ubiquitously expressed makes these toxins and their mutant variants attractive for modulation of immune cell functions. Ctx/Etx and CtxB/EtxB exhibit stimulatory effects on T and B cells, independently of their antigen specificity. Both Ctx/Etx and CtxB/EtxB were shown to differentially modulate the expression of costimulatory molecules and the spectrum of produced cytokines in DC and macrophages, thereby affecting their antigen presenting functions and polarization of T cell responses [120, 126]. While Ctx stimulates predominantly T cells and the production of Th2-associated cytokines [127], Etx triggers production of both Th1 and Th2 cytokines and several antibody subclasses [128]. Ctx/Etx induce activation and isotype differentiation of B cells [115, 129, 130]. In contrast to the stimulatory effects, both CtxB/EtxB induce apoptosis of $CD8^+$ T cells [115, 131]. Furthermore, CtxB and EtxB with conjugated antigens were shown to induce oral tolerance with exceptionally high efficiency [132] and in experimental models suppress the development of autoimmune diseases like experimental autoimmune encephalomyelitis [133, 134], collagen-induced arthritis [135], diabetes [136] or experimental autoimmune uveoretinis [137]. The tolerizing properties of CtxB have been also examined in other immunopathological conditions including IgE-mediated allergies [138], infection-induced tissue-damaging inflammation [139, 140] or graft rejection reactions [141]. These finding have been recently extended to patients with Behcet's disease in a proof-of-concept clinical trial [126, 142].

CRY1AC TOXIN OF *BACILLUS THURINGIENSIS*

Crystal (Cry) proteins are produced by entomopathogenic bacteria *B. thuringiensis* during the sporulation phase in the form of parasporal inclusions. These proteins display a cytotoxic activity against insects such as Lepidoptera, Diptera, Hymenoptera or Coleoptera and also against other invertebrates such as Nematodes [143]. The Cry proteins comprise at least 50 subgroups with more than 200 members. Most of them are globular molecules containing three structural domains connected by single linkers [144, 145]. The N-terminal domain (domain I) is responsible for membrane insertion and pore-formation [145]. Domain II is the receptor binding domain, whereas domain III seems to be important for stability of the toxic fragment [146] as well as for regulation of the pore-forming activity [147]. Upon ingestion by insect larvae the protoxins (~130 kDa) are solubilized in the alkaline environment [148] of the gut and cleaved by the gut proteases yielding 60-70 kDa protease resistant proteins [149]. The activated toxin then binds to specific receptors on the midgut epithelium columnar cells [150, 151]. To date at least four different receptors for Cry1A proteins have been described in insect: a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase N, a GPI-anchored alkaline phophatase and a 270 kDa glycoconjugate [152-155]. It is widely accepted that Cry toxins lyse midgut epithelia cells by forming pores [156]. However, it has been recently suggested that toxicity could be related to G protein-mediated apoptosis following receptor binding [157].

Cry proteins being highly specific to their target insect were found to be innocuous to humans [143, 158]. Accordingly, no mammalian receptor for Cry proteins has been identified so far. Cry genes have been cloned to bacteria and plants to generate biopesticides to control defoliator pests in forestry or to produce transgenic insect-resistant crop such as cotton [159]. Similarly, Cry proteins were exploited as means to control mosquitoes [143, 160]. Most of the immunological studies of Cry proteins have been focused on Cry1Ac protein. According to an old report, insecticidal crystals of *B. thuringiensis* boosted immune response to sheep red blood cells in mice [161]. The same authors have also shown that crystalline Cry proteins exhibited an antitumour activity against Yoshida ascites sarcoma which was caused possibly by the enhancement of general immunity in rats [162]. Recombinant soluble Cry1Ac protein is a potent systemic and mucosal immunogen inducing a strong anti-Cry1Ac antibody response in mice [163-166]. Cry1Ac protein also potentiates antibody responses to co-administered antigens such as hepatitis B surface antigen and bovine serum albumin [164] as well as to polysaccharides of *Streptococcus pneumoniae* [167] with a similar potency as cholera toxin. However, the effects of Cry1Ac on the antibody responses are not consistent and seem to be dependent on the immunization route and the type of antigen [164, 168]. Further study showed that Cry1Ac not only induces local production of specific IgG, but also increases the numbers of epithelial cells containing IgG and stimulates FcRn expression in the intestinal epithelia of adult mice [169].

The N-terminal region of Cry1A toxins was found to be responsible for the immunogenicity [170], therefore its potential as a carrier was investigated. Cry1A mutants carrying a diphtheria toxin epitope were able to enhance specific anti-diphtheria and anti-Cry antibody production and to modulate the ratio of IgG subclasses [171]. Cry1A proteins also induced cell-mediated immune responses. The mutated Cry1Aa8 toxin enhanced production of cytokines and predominantly stimulated IFN-γ in spleen cell cultures which suggests that amino acid substitution in domain I principally affects toxin ability to induce Th1 cytokines in lymphocytes

[172]. Interestingly, Cry1A-induced production of cytokines was inhibited by GalNAc [172]. This indicates that GalNAc of unknown receptors on the cell surface of immune cells is important for the binding of Cry1A toxins and this may initiate the induction of cellular response in mice.

Cry1Ac has been also evaluated for the ability to confer protective immunity in a mouse infection model of *Naegleria fowleri* meningoencephalitis, a fatal acute infectious disease initiated at the nasal mucosa [173]. The intranasal coadministration of Cry1Ac with amoebal lysates completely protected mice challenged with a lethal dose of amoebae, whereas immunization with amoebal lysate alone provided 60 % of protection. It has been found that mucosal but not serum antibody levels seem to be related to the protection against *N. fowleri* infection. The increased IgA response induced at nasal mucosa by immunization probably impedes both amoebic adhesion and subsequent invasion of the parasite to the nasal epithelium [174, 175]. Interestingly, administration of Cry1Ac alone elicited similar protective immunity against *N. fowleri* as mice immunized by amoebal lysate alone, which indicates that Cry1Ac protoxin can stimulate innate immune mechanisms [174].

Cry proteins exhibiting adjuvanticity, innocuity to mammalian cells and cheap production [143, 158] represent an interesting alternative to cholera toxin or heat-labile enterotoxin whose toxicity and elevated production cost limit their application in human vaccines [176]. On the other hand, caution must be exercised as it was shown that exposure of farm workers to *B. thuringiensis* sprays led to allergic skin sensitization and induction of IgE and IgG antibodies [177].

DIPHTHERIA TOXIN OF *CORYNEBACTERIUM DIPHTHERIAE*

Diphtheria toxin (DT) of *C. diphtheriae* is one of the strongest toxins known and by its cytotoxic properties causes a serious respiratory disease. DT is secreted only if the bacterium is infected with a specific corynephage possessing *tox* gene [178]. Diphtheria toxin is a 535 amino acids long protein which consists of three main domains: the catalytic domain C corresponding to fragment A (1-193 aa), the translocation domain T (194-481 aa), and the C-terminal receptor-binding domain R (482-535 aa) corresponding to fragment B [179-181]. DT undergoes proteolytical cleavage by cell-associated proteases [182] prior to binding to the complex of heparin-binding epidermal growth factor (EGF)-like precursor and CD9 receptors [180, 183]. After binding and internalization via clathrin-dependent endocytosis DT unfolds in the acidic environment of endosomes [184] and the disulfide bond between cysteines 186 and 201 is reduced separating two fragments (A and B) of the toxin. The enzymatic A fragment then translocates into the cytosol through a channel made by the T domain [185] where it ADP-ribosylates the elongation factor (EF) 2 [186, 187], thereby causing apoptosis of host cells [188].

Detoxified DT is used for the universal immunization against diphtheria. In addition, non-toxic mutants of DT such as CRM197 [189] containing a single amino acid change in the catalytic domain [190] and CMR9, which has a single amino acid change in the B fragment [191], have been used as carriers in glycoconjugate vaccines to increase immune responses against vaccine antigens from a variety of bacterial pathogens including *Haemophilus influenzae* [192], *Neisseria meningitidis* [193], *Streptococcus agalactiae* [194] or *Streptococcus pneumoniae* [195]. Many of such glycoconjugated vaccines are now actively used for immunization, which led to a dramatic reduction of invasive infections due to *H. influenzae* type b and *S. pneumoniae* in children [196]. DT was also used as a delivery vector for various antigens [197, 198]. DT toxoid carrying epitope of ovalbumin ($OVA_{257-264}$) was effectively delivered into the DT receptor (DTR)-expressing DC and stimulated even more robust OVA-specific $CD8^+$ T cell proliferative response in CD11c-DTR transgenic mice than antigen carrying, detoxified anthrax toxin [199].

An interesting approach to the selective eradication of HIV-infected cells was joining a catalytic A part of DT to *cis*-acting control elements unique to HIV, whose expression is dependent on the HIV regulatory proteins Tat and Nef [200]. Similarly, recombinant DT-based molecules fused with human CD4 (DAB_{389}CD4) which binds gp120 Env of HIV, or to IL-2 (DAB_{486}IL-2 and DAB_{389}IL-2) were shown to target and kill HIV-infected cells [201-205]. Moreover, DAB_{389}IL-2 showed promising results in the treatment of autoimmune diseases, such as psoriasis, rheumatoid arthritis or insulin-dependent diabetes mellitus [206, 207]. The cytotoxic potency of DT was exploited for the generation of immunotoxins which will be described further. The enzymatically active A fragment of DT (DTA) can be used as an effective tool for the cancer gene therapy [208] as plasmids carrying DTA under the control of tumor-specific promotors can be expressed in cancer cell lines, thereby mediating direct cytotoxicity [209]. The therapeutic vector DTA-H19 was tested successfully in mice for the treatment of bladder cancer [210]. Moreover, DT showed a great potential as a candidate for ovarian cancer therapy in humans as the intratumoral injection of DTA-H19 into ectopically developed tumors caused 40 % inhibition of tumor growth in mice [211]. Such vectors can be delivered by nanoparticles to target cancer cells [212].

EXOTOXIN A OF *PSEUDOMONAS AERUGINOSA*

Exotoxin A is one of the toxins secreted by *P. aeruginosa* which can act as an oportunistic bacterial pathogen causing nosocomial infections or infections in immunocompromised individuals. *P. aeruginosa* infection can cause serious complications in ventilator-associated pneumonia and cystic fibrosis patients. *Pseudomonas* exotoxin A (PE) is a 613 amino acids long protein which is composed of three functional domains [213, 214]: the binding domain Ia (1-252 aa), the translocation domain II (253-364 aa) and the cytotoxic, enzymatically active domain III (400-613 aa). The function of domain Ib (365-399 aa) has not been investigated so far [215]. The earliest step of PE action is the removal of C-terminal lysine residue, presumably by plasma carboxypeptidases [216] uncovering the C-terminal REDL sequence. PE binds to CD91 receptor (α2-macroglobulin receptor/low-density lipoprotein receptor-related protein) expressed by many different cell types [217]. The toxin is internalized mainly through clathrin-dependent endocytosis, but also through caveosomes, into early endosomes [218]. In the acidic environment of endosomes PE is cleaved by furin and

the disulfide bond between cysteines 265 and 287 is reduced which separates the N-terminal 28 kDa and C-terminal 37 kDa fragments [219]. The C-terminal amino acids REDL then bind to the KDEL receptor sequence which allows the transport of enzymatically active C-terminal fragment of the toxin from Golgi apparatus to endoplasmic reticulum [220]. The active part of the toxin is transported from endoplasmic reticulum possibly via Sec61 complex [221] into the cytosol, where it ADP-ribosylates EF2 [222], which leads to the inhibition of protein synthesis and subsequently to apoptosis [223], most likely by activation of the caspase 8-dependent pathway [224].

PE has been intensively studied for immunological purposes as a vector for antigen delivery, as a carrier for co-administered antigens, and most importantly, as an immunotoxin for cancer immunotherapy. Using PE for antigen delivery requires its detoxification [225, 226]. The immunization of mice with non-toxic mutant PEΔ576-613, lacking the catalytic domain III, protected against PE intoxication [226]. Mutant PE variant PEΔE553 carrying ovalbumin polyepitope ($OVA_{204-386}$) elicited $CD8^+$ T cell-mediated protective immunity against lethal tumor challenge [227]. Similarly, PE lacking domain III and carrying peptide epitopes from influenza A matrix protein or nucleoprotein were shown to sensitize target cells for lysis by peptide-specific human and murine CTLs [228]. PE fusion proteins have proven to be effective also for antigen delivery to MHC class II pathways [229]. Here, human proinsulin linked to PE toxin (PE-PI) lacking the catalytic domain III stimulated specific $CD4^+$ T cells *in vitro*. Whereas processing of PE-PI appeared to follow the classical endocytic pathway, the processing of detoxified PE carrying an HLA-A*0201-restricted melanoma-specific CTL epitope (PE-$gp100_{280-288}$) occured independently of the proteasome pathway, however, still sensitizing target cells to lysis by specific CTLs [229]. Interestingly, these fusion proteins were more stable in human plasma than the synthetic peptides alone. Mice immunized with a DNA vaccine containing PE domain II fused with a model tumor antigen E7 from HPV16, or with a recombinant protein vaccine containing PE lacking domain III fused with E7, generated strong specific $CD8^+$ T cell-mediated immunity which conferred protective and therapeutic immunity against the E7-expressing tumors [230, 231].

PE-based immunotoxins were not only used for cancer immunotherapy but also as anti-viral agents. PE lacking domain I fused with CD4 (CD4-PE) was shown to kill HIV-infected cells expressing Env on their surface [232]. In peripheral blood mononuclear cells and monocyte-derived macrophages the immunotoxin inhibited spreading infection of primary HIV isolates [233, 234]. Despite promising results in preclinical studies, CD4-PE immunotoxin failed to show any anti-viral or immune enhancing effects in Phase I clinical trial conducted in pre-HAART (Highly Active Anti-Retroviral Therapy) era 16 years ago [235, 236]. No additional clinical trials have been conducted since [237]. Later, an improved Env-targeted immunotoxin 3B3(Fv)-PE38 was contructed where PE was combined with a single chain Fv (scFv) of an high affinity anti-gp120 Env antibody [238]. 3B3(Fv)-PE38 showed a greater potency in killing HIV-infected cells and in the inhibition of infection spreading, as well as no hepatotoxicity, than CD4-PE [238, 239]. When 3B3(Fv)-PE38 was combined with agents that block viral replication (HAART drugs) a strong long-term suppression of viral loads was observed in human tissue implant [240]. Based on these results, the Env-targeted immunotoxin concept have been proposed to reconsider for depletion of persisting HIV-infected cell reservoirs as a complementation of HAART [237, 241].

PE-derivatives were also shown to be potent mucosal immunogens [226, 242]. It was found that PE with the deletion of a glutamic acid at position 553 (PEΔE553) carrying antigenic epitope from gp120 of HIV or from the VP1 capsid protein of foot and mouse disease virus induced mucosal and systemic antigen-specific antibody response [243, 244]. Over the last 20 years, PE have been studied as a carrier protein in glycoconjugate vaccines to help to increase the immune response to various poorly immunogenic bacterial polysaccharides from *Staphylococcus aureus* [245], *P. aeruginosa* [246] and *Klebsiella pneumoniae* [247]. Despite very positive data from rodent models and *in vitro* studies, all four clinical trials failed to prove a significant protection over the placebo control raising concerns about the benefit of PE in glycoconjugate vaccines [248].

IMMUNOTOXINS BASED ON *PSEUDOMONAS* EXOTOXIN A AND DIPHTHERIA TOXIN

Pseudomonas exotoxin A (PE) and diphtheria toxin (DT) have been successfully used as immunotoxins for cancer immunotherapy which was summarized in two excellent reviews [215, 249]. Immunotoxins are molecules consisting of a protein toxin and a ligand which is usually an antibody, growth factor or cytokine. On the surface of the target cell, the ligand binds to a tumor-associated antigen which then delivers the toxin into the cell cytosol, thereby causing the cell death. The first immunotoxins were produced by chemical coupling of antibodies to a whole or truncated form of PE or DT. However, the poorly defined heterogenous composition, lack of stability and specificity of these constructs led to a new generation of immunotoxins, which could be easily prepared by recombinant techniques in *E. coli*. The most common form of truncated PE toxin PE38 is composed of amino acids 253-364 and 381-613 of PE missing its binding domain Ia and part of Ib domain [250]. The most common truncated form of DT is DT388 or DAB389 with first 388 amino acids of the toxin [251]. PE-based immunotoxins were shown to be effective mainly in hematological malignancies. In solid tumors, however, they failed to provoke partial or complete remissions. One reason may be the accessibility of tumor cells for the immunotoxin. A large number of PE- and DT-based immunotoxins directed against various tumor-associated antigens were constructed and tested in preclinial trials. Some immunotoxins were evaluated in clinical Phase I trials and are summarized in (Table **3**) [215, 249, 252]. However, some immunotoxins still exhibited a lack of specificity and also side effects caused by a non-specific binding leading to hepatotoxicity or vascular leak syndrom, which were often dose-limiting. Their immunogenicity represents another crucial property of immunotoxins as a high number of patients developed anti-immunotoxin antibodies. On the other hand, immunotoxins displayed major effects on immune responses after the failure of standard chemotherapy suggesting that they can be useful tool for treatment of

Table 3. Diphtheria Toxin and *Pseudomonas* exotoxin A-based Immunotoxins that have been Evaluated in Clinical Trials

Immunotoxin	Antigen[a]	Cancer Type	Ref.
Diphtheria Toxin			
Denileukin Diftitox	IL-2R	Cutaneous T cell lymphoma, Chronic lymphocytic leukemia Non-Hodgkin lymphoma	[255, 256, 257]
DT388-GM-CSF	GM-CSFR	Acute myeologenous leukemia	[258]
DT388-IL3	IL-3R	Acute myeologenous leukemia Myelodysplastic syndrom	[259]
TF-CRM107	TFR	Glioma	[260]
$DAB_{389}EGF$	EGFR	Carcinoma	[261]
***Pseudomonas* exotoxin A**			
BL22	CD22	Hairy cell leukemia, Chronic lymphocytic leukemia Non-Hodgkin lymphoma	[262, 263]
LMB-2	CD25	Non-Hodgkin lymphoma Leukemia	[264, 265]
OVB3-PE	Ovary	Ovarian cancer	[266]
LMB-1	LeY	Adenocarcinomas	[267]
SGN-10 (BR96(scFV)-PE40	LeY	Adenocarcinomas	[268]
LMB-7 (B3(Fv)-PE38) LMB-9 (B3(desFv)-PE38)	LeY	Carcinoma	[269]
IL-4 (8-37)-PE38KDEL	IL-4R	Glioma	[270]
NBI-3001	IL-4R	Glioma, solid tumors	[271, 272]
Erb-38	ERBB2	Breast, esophageal cancer	[273]
scFv(FRP5)-ETA	ERBB2	Breast, colon cancer, melanoma, Solid tumours	[274, 275]
TP38	EGFR	Glioma	[276]
TP40	EGFR	Bladder cancer	[277]
SS1P	Mesothelin	Mesothelioma, ovarian, pancreatic cancer	[278]
IL13-PE38QQR	IL-13R	Glioma, renal cell carcinoma	[279, 280]

[a]Abbreviations: GM-CSFR, granulocyte-monocyte colony stimulating factor receptor; TFR, transferrin receptor; EGFR, endothelial growth factor receptor; LeY, lewis-related antigen; ERBB2, human epidermal growth factor receptor 2 (also HER2/neu)

minimal residual disease. The success of immunotoxins against hematological malignancies is emphasized by the approval of a recombinant DT-based immunotoxin DAB(389)IL-2 (denileukin difitox) for cutaneous T cell lymphoma which is marketed under the brand name ONTAK. Similarly, PE-based immunotoxin HA22, an improved version of BL22, is headed for more advance trials [215, 246, 252-254].

PERFRINGOLYSIN O OF *CLOSTRIDIUM PERFRINGENS*, INTERMEDILYSIN OF *STREPTOCOCCUS INTERMEDIUS* AND PNEUMOLYSIN OF *STREPTOCOCCUS PNEUMONIAE*

Perfringolysin O (PFO) of *C. perfringens*, Intermedilysin (ILY) of *S. intermedius* and pneumolysin (PLY) of *S. pneumoniae* are cholesterol-dependent cytolysins which exhibit a high level of identity at the primary structure level. These toxins form large, homo-oligomeric pores in cellular plasma membrane and their cytolytic activity is dependend on the presence of membrane cholesterol. In contrast to PFO and PLY, ILY exhibits a restricted specificity for human cells expressing the CD59 molecule [281, 282]. In pathogenesis of *C. perfringes*-induced gas gangrene PFO seems to primarily kill macrophages and to aid bacteria to escape from the macrophage phagosome [283]. The cytolytic activity of PFO was used to deliver DNA complexes using biotin-strepavidin bridge for the expression into the cells as an alternative to viral DNA vectors [284]. Recently, a novel recombinant Bacillus Calmette-Guerin (BCG) strain expressing PFO together with the key immunodominant antigens of *M. tuberculosis* has been described [285]. The vaccination of mice

and guinea pigs resulted in enhanced immune responses and prolonged the survival after the challenge with a hypervirulent *M. tuberculosis* strain HN878.

Intermedilysin (ILY) is an important factor of *S. intermedius* to form abscesses. ILY was shown to target polymorphonuclear cells [286] and to increase host cell susceptibility to a complement-mediated lysis by blocking the CD59-binding site for complement proteins CD8α and CD9 [287, 288]. Exploiting the complement regulators including CD59 as a part of the viral envelope represent a strategy of many viruses to resist a complement-mediated virolysis. Therefore the receptor-binding domain 4 of LLY [289] was used to create a novel therapeutic agent against HIV which specifically rendered HIV virions susceptible to complement-mediated lysis which was activated by specific antibodies in serum of patients [290]. Moreover, the domain 4 of LLY bound to erythrocytes was shown to deliver a coupled anti-carcinoembryonic antigen monoclonal antibody to antigen-positive cell line which suggested that LLY could be used as a cell membrane adaptor module for the drug delivery [289].

Pneumolysin (PLY) is a key virulence factor of *S. pneumoniae* which activates a large number of genes and multiple signal transduction pathways in host cells. Cytolytic effects contribute to lung injury and neuronal damage, while pro-inflammatory effects compound tissue damage. PLY induces apoptosis in macrophages via binding Toll-like receptor 4 (TLR4) and this binding is also important for the resistance to pneumococcal infection [291]. The detoxified PLY mutant, also as a part of pneumococcal conjugate vaccine, conferred protection against bacterial challenge in mouse infection model [292, 293]. Additionally, PLY genetically fused with protein antigen PsaA of *S. pneumoniae* can act as an adjuvant to induce mucosal as well as systemic humoral response, even at nanogram quantities of the conjugated protein [294]. The toxic activity of PLY was not essential for the adjuvanticity but contributed to the magnitude of the immune response generated. Unfortunately, the immunization of mice with PsaA-PLY did not confer protective immunity against *S. pneumoniae*. This recent study showed that PLY can be exploited as a novel mucosal adjuvant to genetically fused protein antigens [294].

PERTUSSIS TOXIN OF *BORDETELLA PERTUSSIS*

Pertussis toxin (PT) is an important virulence factor produced exclusively by *B. pertussis*. PT is believed to be the main cause of clinical symptoms characteristic of whooping cough. PT composes of enzymatically active A subunit (S1) and five B subunits (S2, S3, two subunits of S4 and S5) which create a ring-like form and are responsible for the toxin binding via glycolipids or glycoproteins containing sialic acid to the surface of mammalian cells [295, 296]. PT utilizes a retrograde transport pathway through Golgi apparatus to endoplasmic reticulum where the A subunit of the toxin dissociates and is translocated presumably through the Sec61 channel across the membrane into the cytosol [297]. In the cytosol, the A subunit ADP-ribosylates several small heterotrimeric G-proteins that regulate the activity of adenylate cyclases which results in accumulation of cAMP within the host cells [298]. In the pathogenesis of *B. pertussis* infection PT targets airway macrophages, inhibits the recruitment of neutrophils and displays multiple suppressive effects on the adaptive immune responses [299-303]. Furthermore, PT promotes Th1 and Th17 inflammatory cytokine upregulation in the airways at the peak of *B. pertussis* infection [303, 304]. Several studies, however, showed that PT possesses adjuvant properties and is able to enhance antibody production as well as Th1 and Th2 responses to co-administered antigens [305-307]. PT was shown to induce maturation and cytokine production in DC via its binding to TLR4/TLR2 receptor [308-311]. More importantly, several studies revealed that the immunostimulatory activity of PT is not only dependent on the ADP-ribosylating capacity of the A subunit, but also on the receptor-binding activity of pentameric B subunits as a mutated PT (PT9K/129G) [312] lacking enzymatic activity still retained similarly potent adjuvant activity [305, 307, 308, 311, 313]. PT9K/129G has been found to induce proinflammatory cytokines in DC [308] that skewed T cells towards Th1/Th17 phenotype [314]. PT-B oligomer seems to utilize T cell receptor to activate T cells directly [315-317]. The binding activity of PT-B alone elicits intracellular signaling events which differ from that induced by the enzymatic activity of PT [201, 308, 311]. These effects typically require 10- or 100-fold higher concentrations of PT than those required for G-protein modification [303]. In addition, PT9K/129G carrying an antigenic epitope from nucleoprotein of LCMV ($NP_{118-126}$) was shown to sensitize target cells to MHC class-I restricted lysis *in vitro* [318]. Interestingly, PT bypassed the classical proteasome-dependent cytosolic pathway for MHC class I-restricted antigen presentation suggesting an alternative route of antigen processing. The safety and immunogenicity of PT9K/129G for the use as a monovalent vaccine or as a component of acellular pertussis vaccine were confirmed in clinical trials [110, 319.]

PT or PT-B were further shown to be effective tools for controling viral infection as it was observed that the PT holotoxin as well as the B oligomer inhibited HIV infection at multiple stages of viral replication *in vitro* [320-322]. Similarly, PT mediated a partial anti-viral effect against LCMV and simian immunodeficiency virus infection of mice and rhesus macaques, respectively [323, 324]. PT9K/129G also exhibited similar anti-HIV effects as the PT-B form [322] and its fusion with CTL epitope of HIV gp120 Env protein induced a strong specific CTL response in mice [325]. In addition, PT-B was able to successfully block viral infection and replication in mice model of HIV infection [326], and both enzymatically inactive forms of PT, PT-B and PT9K/129G, inhibited HIV-1 replication in human lymphoid tissue *ex vivo* [201]. It has been found that PT-B inhibits cellular events mediated by the HIV Tat transactivating protein like TGF-β induction in natural killer cells or apoptosis of neuronal cells [327-329]. Moreover, PT-B was shown to inhibit IL-6-mediated induction of HIV in chronically infected promonocytic cells [201, 303, 330].

Adjuvant activity of PT, on the other hand, also accounts for the exacerbation of experimental autoimmune encephalomyelitis, a rodent model of multiple sclerosis in humans [331]. It has been suggested that PT increases permeability of the blood-brain barrier [310, 332], decreases a number of T regulatory cells [333] or induces Th17 cell responses [334].

SHIGA TOXIN OF *SHIGELLA DYSENTERIAE* AND SHIGA-LIKE TOXIN OF *ESCHERICHIA COLI*

Shiga toxin produced by *S. dysenteriae* and Shiga-like toxins (also verotoxins) produced by certain serotypes of *E. coli* (STEC; Shiga toxin-producing *E. coli*), and some other bacteria, are responsible for serious medical conditions like dysentery, hemorrhagic colitis or hemolytic uremic syndrom. Shiga toxins can be classified into different groups [335]. Shiga toxin (Stx) of *S. dysenteriae* is almost identical to *E. coli* Shiga-like toxin 1 (Stx1), also called VT1 (verotoxin 1). There are also several variants of Shiga-like toxin 2 (Stx2), also called VT2 (verotoxin 2), which have a similar structure, but display different immunological properties and effects on target cells than Stx1 [336].

All toxins belong to the AB family of protein toxins. The enzymatically active A part consisting of A1 and A2 domains is non-covalently associated with a non-toxic homopentameric B subunits which are responsible for toxin binding to globotriose-ceramide receptors (Gb3; CD77) expressed on the surface of sensitive cells [337]. The toxin is transported in a retrograde fashion from the plasma membrane via endosomes into Golgi apparatus and endoplasmic reticulum [338, 339]. The enzymatically active A part of the molecule, proteolytically activated by the host cell protease furin, is then translocated into the cytosol of the host cell where it inhibits protein synthesis by modifying a conserved residue of 28S rRNA, thereby causing the cell death [337, 340]. Shiga toxins stimulate production of cytokines such as IL-1, IL-6, IL-8, TNF-α or GM-CSF in different cell types via activation of various mitogen-activated protein kinases [341-343]. Lee *et al.,* first reported that the non-toxic B subunit of Shiga toxin (StxB) carrying an epitope from a model tumor antigen, Mage 1, could be presented by human peripheral blood mononuclear cells in an MHC class-I restricted manner to Mage 1-specific CTLs [344]. Similarly, Shiga-like toxin 1 of *E. coli* carrying either N- or C-terminally fused peptide derived from influenza virus matrix protein was shown to generate a $CD8^+$ T cell response *in vitro* [345]. No additional adjuvant was needed for the induction of specific anti-tumor CTLs in mice after immunization with StxB carrying a mouse tumor epitope [346]. The same research group later showed that vaccination with StxB carrying chemically coupled ovalbumin primed specific anti-OVA CTLs and Th1-polarized responses, and induced IgG2a antibodies [347]. Similarly, Choi *et al.,* observed that the oral immunization with StxB linked to a fragment of the immunogenic rotavirus nonstructural protein 4 induced protective humoral and cellular responses in mice [348]. It seems that StxB delivers antigen directly into the DC *in vivo* and induces a robust and long-lasting specific $CD8^+$ T cell response which confers both prophylactic as well as therapeutic tumor protection [349]. This could be further improved by the addition of α-galactosylceramide [350], whose adjuvant activity lies among others in the stimulation of DC maturation [351]. Even though Vingert *et al.,* observed no maturation of bone marrow-derived DC after *in vitro* incubation with StxB [349], others reported an increased expression of costimulatory and MHC class II molecules on DC after a nasal administration or after *in vitro* incubation with Stx1B [352, 353]. Furthermore, Stx1B was shown to induce mixed Th1 and Th2 type $CD4^+$ T cells and enhanced the antibody response to a co-administered antigen [353].

Interestingly, Stx1 was also reported to exhibit an antiviral activity [354, 355]. Specifically, StxA coupled to the human CD4 domain responsible for binding to Env protein of HIV selectively killed chronically infected 8E5 T cells [354, 356]. The non-toxic Stx1A also reduced the expression and replication of bovine leukemia virus and bovine immunodeficiency virus [357, 358], and Stx1-producing *E. coli* were shown to mitigate bovine leukemia virus infection in experimentally infected sheep [359].

Shiga toxin receptor Gb3 was shown to be expressed on malignant, even metastasizing cells [360-363]. This can be exploited for the diagnosis of cancer as it was shown that StxB could reach Gb3-expressing digestive tumors in animal models as well as human colorectal tumors and their metastasis [364, 365]. Recently, a prodrug composition using topoizomerase I inhibitor SN38 coupled to StxB was designed in order to specifically target cancer cells [366]. It has also been suggested that Stx can be therapeutically used against some forms of cancer without any modification as the intratumoral injection of Shiga toxin could inhibit tumor growth in mice [367-370].

HEMOLYSIN A OF *ESCHERICHIA COLI*

The bacterial protein toxin α-hemolysin (HlyA) secreted mainly by uropathogenic *E. coli* is a single 107 kDa polypeptide that belongs to the RTX family of toxins. The *E.coli* HlyCABD operon comprises the genes *hly*C and *hly*A which direct the synthesis of an active toxin, and *hly*B and *hly*D which determine its secretion [371]. HlyA is synthesized as a non-toxic prohemolysin (proHlyA), which is further activated to a mature toxin by the co-synthesized fatty acid acyltransferase HlyC [372]. The HlyA export machinery is the best characterized type I secretion system which consists of three components HlyB, HlyD and TolC [371, 373, 374]. While the inner membrane proteins HlyB and HlyD are specific components of the transport apparatus of HlyA, the third component, TolC is a multifunctional protein located in the bacterial outer membrane [372, 375]. HlyA is secreted across both membranes without the aid of an N-terminal signal sequence [376]. At its C-terminus, HlyA carries a secretion signal of about 50-60 amino acids in length (termed HlyAs), which is recognized by the HlyB/HlyD/TolC-translocator, promoting direct secretion of the entire protein into the extracellular medium without the formation of periplasmic intermediates [377]. The HlyAs appears to be necessary and sufficient for the secretion of HlyA [378]. HlyA targets endothelial cells and cells of the immune system, particularly granulocytes and monocytes. At high concentration, HlyA causes target cell lysis by generating cation-selective pores [379]. HlyA was shown to alter calcium signaling and promote proinflammatory cytokine release (IL-6 and IL-8) in renal tubular cells [380]. The HlyA secretion system can be used to secrete heterologous proteins of various size and origin covalently linked at their C-terminus to HlyAs which has been mainly applied to immunological and vaccine research [372, 381-384]. This system was used in the immunotherapy of tumors [385, 386] or for co-expression and co-delivery of active cytokines [387]. Moreover, as HlyAs itself is a weak immunogen, hemolysin-fusion

proteins were successfully used to produce polyclonal and monoclonal antibodies [388] or to detect immunodominant antigens of *Helicobacter pylori* [389]. The most important application of the HlyA secretion system is the delivery of heterologous antigens for antigen presentation via attenuated live bacterial carrier strains which allows induction of humoral and/or cell-mediated immune responses (Table **4**) [372]. So far, the HlyA secretion system is the only Type I protein exporter machinery, which has been explored as a secretory expression system in a variety of Gram-negative bacteria including different *Salmonella* serotypes, *Shigella* sp. or *V. cholerae* [382, 383] and has proven its value in a wide range of animal systems, including mice, rabbits and cattle. However, there are a number of limitations depending on the nature of the heterologous protein which influence the secretability and the secretion efficiency. Similarly, the growth-dependent regulation prevents a continuous expression of foreign proteins under *in vivo* conditions [390]. The most important issue is the efficacy of the system for use in humans. To date, Vivotif is the only licensed attenuated live oral vaccine against typhoid fever for human use [391]. The main constituent of this oral vaccine is *Salmonella enterica* Serovar Typhi Ty21a which has also been widely used for the delivery of heterologous antigens via the hemolysin secretion system and therefore may be a potential candidate for clinical trials. Recently, this strain has been improved by the addition of a plasmid encoding *rfaH* gene which enhanced the antibody response against heterologous HlyA antigen of Ty21a after immunization of mice [392].

LISTERIOLYSIN O OF *LISTERIA MONOCYTOGENES*

Listeriolysin O (LLO) is a primary virulence factor of *L. monocytogenes*, which causes listeriosis, a severe systemic disease that affects immunocompromised individuals and pregnant women. LLO is a 529 amino acids long protein of the cholesterol-dependent cytolysin family. It is selectively activated within the acidic phagosome of cells that have phagocytosed *L. monocytogenes* [407]. It lyses the phagosome and aids bacteria to escape into the cytosol, where they can grow intracellularly and eventually spread to neighbouring cells [408, 409]. Moreover, LLO has recently been shown to promote bacterial replication also in the phagosome [410]. LLO contains an N-terminal PEST-like sequence which is crucial for the survival of *L. monocytogenes* in the host [411]. It also controls LLO synthesis during the cytosolic growth of bacteria [412, 413]. The PEST sequence is usually found in eukaryotic proteins targeted for rapid degradation in the ubiquitin-proteasome pathway [414]. Therefore LLO represents a particularly attractive tool for vaccine delivery due to its own efficient processing into an MHC class I epitope [415].

LLO has been used to enable delivery of purified protein antigens or antigens encapsulated in liposomes into the cytosol of APCs which elicited $CD8^+$ T cell responses [416, 417]. LLO has been also co-expressed in a wide variety of attenuated live bacterial vaccine strains such as *B. subtilis*, *B. anthracis, M. bovis* Bacillus Calmette-Guerin (BCG), *S. typhimurium aroA*, *E. coli* and *L. lactis* and proven to promote access to the cytosol of APCs for bacterial as well as heterologous antigens and DNA vaccines which resulted in cytosolic processing and presentation on MHC class I molecules, leading to $CD8^+$ T cell stimulation [418, 419]. Tuberculosis vaccine strain *M. bovis* BCG expressing LLO has been tested in clinical trials for its vaccine efficacy [420]. In addition, a variety of viral and tumor antigens fused with LLO and expressed by *L. monocytogenes* have been shown to generate antigen-specific $CD4^+$ and $CD8^+$ T cell responses and induce anti-tumor immunity in mice (Table **5**) [421]. LLO fusion to tumor antigens in viral vectors [422] or DNA vaccines [423] also enhances vaccine efficacy which suggests that LLO exhibits an adjuvant potential.

Neeson *et al.,* have shown that the administration of a recombinant LLO conjugated with lymphoma immunoglobulin idiotype (Id-LLO) induced a potent humoral and Th1 polarized T cell-mediated immune response [424]. However, the adjuvanticity of LLO is only partially understood. It seems that LLO induces DC maturation and the production of proinflammatory cytokines such as IL-12, IL-18 and IFN-γ [425]. LLO also inhibits a Th2 immune response in the experimental rhinitis induced by ovalbumin by shifting the differentiation of antigen-specific T cells to Th1 cells [425, 426]. Similarly, *L. monocytogenes* expressing LLO-E7 was shown to enhace DC maturation leading to a more robust anti-tumor response in comparison to *L. monocytogenes* expressing only E7 [423]. It has been also found that LLO suppresses $CD4^+CD25^+$ T regulatory cell function *in vivo* [427]. Interestingly, LLO has recently been exploited as a component of an immunotoxin [428]. Here, LLO coupled with Fv fragment of the monoclonal antibody B3 against the tumor antigen Lewis Y was shown to eliminate antigen positive MCF7 cells.

The combination of the ability of *L. monocytogenes* to introduce antigens into MHC class I and II processing pathways coupled with the fact that LLO seems to be a potent immunogenic molecule may have important implications for anti-tumor vaccination strategies in humans. Recently, Advaxis Inc., a New Jersey-based biotechnology company showed in a phase I clinical trial using its *L. monocytogenes*-based construct *Lm*-LLO-E7 expressing the tumor antigen E7 of HPV16 (ADXS11-001, formely Lovaxin C) that vaccination was safe for use in patients with late stage invasive cervical carcinoma [429].

BACTERIAL VECTORS FOR IMMUNOTHERAPY

The use of bacteria as vectors for immunotherapy represents an attractive vaccination strategy that has several advantages. Bacteria can be engeneered to carry various heterologous proteins, plasmid DNA or mRNA vaccines, their pathogenicity can be genetically attenuated and they can be easily produced in large quantities. Bacterial vectors can be administered orally or nasally, and their intrinsic adjuvant properties stimulate mucosal and systemic immune responses. Mainly, derivatives of pathogenic food-related bacteria like *Salmonella* sp., *Listeria*, *Yersinia* or *Shigella* as well as non-pathogenic commensal bacteria including *Lactococcus lactis*, *Lactobacillus* spp. or *Streptococcus gordonii*, have been evaluated as live vaccines, however, none of them has reached the market yet [442]. Moreover, some other bacteria have been exploited as vaccine carriers such as *Bordetella bronchiseptica*, *Erysipelotrix rhusiopathiae*, *Mycobacterium bovis*, *Brucella abortus* [442], *Helicobacter pylori* [443], *Bacillus subtilis* [444] or *V. cholerae* [121].

Table 4. List of Antigens Fused With Truncated α-hemolysin (HlyA) of *E.coli*

Antigen Fused to HlyAs	Origin	Bacterial Carrier	Immune Response in Animal Model (Model; CMR)[a]	Protection in Animal Model[b]	Ref.
Bacteria					
OspA	*Borrelia burgdorferi*	*E. coli* TB1	ND	ND	[393]
Diphtheria toxin	*C. Diphtheriae*	*S. Typhi* CVD 908-*htrA*	BALB/c; ND; Ab response	ND	[394]
Listeriolysin O	*L. Monocytogenes*	*S. Typhimurium aroA* SL7207	C57BL/6; CTL, $CD4^+$ T cells	+	[395, 396]
p60					
Superoxide dismutase					
Ag85b	*M. Bovis BCG*	*S. Typhimurium aroA* SL7207	C57BL/6; TNF-α, IFN-γ production	+/-	[397]
ESAT-6	*M. Tuberculosis*	*S. Typhimurium aroA* SL7207	BALB/c; T cell proliferation, IFN-γ production	+/-	[398]
PagC SlyA	*S. Typhimurium*	*E. coli* K5	ND	ND	[388, 399]
StxB	*S. Dysenteriae*	*S. Typhimurium aroA* SL3261 *S. Flexneri aroD*	BALB/c; CTL, $CD4^+$ T cells, Ab response Neutralizing Ab	ND ND	[400] [401]
Streptokinase	*Streptococcus equisimilis*	*E. coli* K5	ND	ND	[402]
Vírus					
Nucleocapsid protein Fusion protein	Measles virus	*S. Typhimurium aroA* SL7207	C3H; T cell proliferation Ab response	+/-	[403]
Parasites					
p67	*Theileria parva*	*Salmonella dublin aroA* SL5631	Cattle; ND; Ab response	+/-	[404]
SSP-2	*Plasmodium falciparum*	*S. Typhimurium aroA* SL3261 *S. Typhi* CVD 908-*htrA*	C57BL/6; IFN-γ production	ND	[405]
Humans					
IL-6		*E.coli* XL-1 Blue/ *S. Typhimurium* X4064	HeLa; B9 mouse hybridoma	ND	[387, 406]
C-raf		*S. Typhimurium aroA* SL7207	C57BL/6; CTL Ab responses	+/-	[386]
Prostate-specific antigen, CtxB		*S. Typhimurium aroA* SL7207	DBA/2; CTL	+	[385]

[a]Abbreviations: Ab, antibody response; CMR, cell-mediated response; CtxB, cholera toxin B subunit.
[b]+, 100% protection; +/-, partial protection; ND, not determined.

Table 5. List of Antigens Fused to Listeriolysin O (LLO) of *L. monocytogenes*

Antigen fused to Listeriolysin O	Origin	Carrier	Immune Response (Model; CMR)[a]	Protection in Animal Model[b]	Ref.
Nucleoprotein (NP)	Influenza	*Lm*-LLO-NP	BALB/c	ND	[430]
		Lm-LLO-NP	BALB/c, C57BL/6; CTL	+	[431]
Prostate specific Antigen	Prostate cancer	*Lm*-LLO-PSA	C56BL/6	+	[432]
E7	HPV-16	*Lm*-LLO-E7 *Lm*-E7	C57BL/6 ; CTL, $CD4^+$ T cells	+	[433]
		Lm-LLO-E7	C57BL/6, CTL	ND	[434]
		Lm-LLO-E7	Human patients	ND	[429]
		Vac-LLO-E7 (Vaccinia)	C57BL/6; CTL	+/-	[422]
Capsid protein 1 (L1)	HPV-16	*Lm*-LLO-L1(1-258) *Lm*-LLO-L1(238-474)	C57BL/6	ND	[435]
High molecular weigh melanoma-associated antigen	Melanoma	*Lm*-LLO-HMW-MAA-C	C57BL/6, BABL/c, FVB/N; CTL, $CD4^+$ T cells	+	[436]
WT1	Wilms tumor Gene 1	*Ec*-LLO-WT1 (*E.coli*) *Ec*-LLO	CTL	+	[437]
Fetal liver kinase-1	Breast cancer	*Lm*-LLO-Flk1	FVB/N; CTL	+/-	[438]
HER-2/neu	Breast cancer	*Lm*-LLO-EC1 *Lm*-LLO-EC2 *Lm*-LLO-EC3 *Lm*-LLO-IC1 *Lm*-LLO-IC2	FVB/N; CTL	+	[439, 440]
Tumor associated Antigens (TAAs)	Breast cancer	*Lm*-LLO-Mage-b(311-660), *Lm*-LLO	C57BL/6; CTL	+	[441]
Lymphoma immunoglobulin idiotype (Id)	Follicular lymphoma	Id-rLLO	C3H/HeN; $CD4^+$ T cells, Ab	+	[424]

[a]Abbreviations: CMR, cell-mediated response; *Lm*, *Listeria monocytogenes* strain; HPV16, human papillomavirus 16; Ab, antibody response.
[b]+, 100% protection; +/-, partial protection; ND, not determined.

In contrast to lactic acid commensal bacteria, *Salmonella* sp., *Listeria*, or *Shigella* can invade and survive in phagocytic as well as non-phagocytic cells, thus facilitating the delivery of antigens for priming peptide-specific $CD4^+$ T cells. However, the confinement of bacteria such as *Salmonella* or *Shigella* within the intracellular compartments complicates endogenous MHC class I-restricted antigen presentation and subsequent $CD8^+$ T cell induction. To circumvent this problem the bacterial type III secretion system (T3SS) has been manipulated to deliver heterologous proteins directly into the cytosol of APCs [445]. In *Salmonella* strains, two virulence-associated T3SS are encoded by separate pathogenicity islands, *Salmonella* pathogenicity island 1 (SPI1) and 2 (SPI2) [446]. A large number of effector proteins such as SptP, SopE2, SseF, SspH, and SifA translocated by the T3SS have been used to serve as carrier molecules for translocation of vaccine epitopes [445, 447-450]. Moreover, the immunization with recombinant *Salmonella* strains secreting antigens conferred protection against lethal viral or bacterial infection in animal models [445, 451, 452]. *Salmonella enterica* serovar *typhimurium* as well as *Listeria monocytogenes* have also been extensively studied as vectors for tumor-associated antigens in cancer immunotherapy [453]. Only a few clinical trials have been conducted so far demonstrating the safety of this system in humans but they also highlighted the need for the generation of strains with lower toxicity and improved colonization properties [454]

Yersinia represent other bacteria utilizing T3SS for pathogenicity. Several studies have described the potential of YopE, a *Yersinia* outer protein secreted by T3SS, to function as a carrier molecule for heterologous antigen delivery [451, 452, 455, 456]. *Y. enterocolitica* expressing the cholera toxin B subunit or CRA protein of *Trypanosoma cruzi* fused to YopE was shown to induce an antibody response against these antigens in mice [457, 458]. In these studies the authors did not use attenuated *Yersinia* strains but rather used *Y. enterocolitica* serotype O:9 strains that are of low viru-

lence in the mouse model. It was demonstrated that also attenuated mutants of the highly mouse-virulent *Y. enterocolitica* serotype O:8 were suitable to function as a live carrier vaccine [459]. Similarly, attenuated *Y. pseudotuberculosis* mutants have been evaluated as live carrier vaccines in the murine listeriosis model [460]. A single oral immunization of mice with recombinant *Y. pseudotuberculosis yopK*-null mutant expressing the YopE translocation domain fused to LLO resulted in an LLO-specific induction of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells which protected mice from a lethal challenge with *L. monocytogenes* [460].

CONCLUSION

Based on the data from *in vitro* as well as *in vivo* preclinical studies, recombinant protein toxins exploited as vectors for antigen delivery were shown to be efficient tools for inhibition of viral infection and immunotherapy of malignant diseases. However, their use in protection against bacterial infections seems to be limited which is most likely determined by their capacity to carry only a small number of protective bacterial antigens. So far, DT and PE-based immunotoxins represent the most promising achievement in the field of bacterial toxin immunotherapy. However, further improvement of immunotoxins is necessary to limit their side effects, to increase their molecular specificity and transport through physiologic barriers, and to enhance their capacity to withstand inactivation by the immune system [215, 249]. The ability of bacterial toxins to bind their specific cellular receptors has also a great potential as a diagnostic tool for profiling of human tumors *in vivo*. Toxin conjugation with a number of detectable moieties including fluorochromes, radionuclides, fluorescent proteins or even magnetic resonance image contrast agents can provide real-time, noninvasive imaging of specific cells or cell-associated enzymatic activity [93, 364, 365]. Several toxins which act as excellent immunological adjuvants exhibit unfortunatelly high toxicity in humans and their production is expensive. Therefore Cry proteins of *B. thuringiensis* represent an interesting alternative due to their innocuity to humans, stable biochemical properties and cheap large-scale production. Nevertheless, further evaluation of effects of Cry proteins on mammalian cells is needed to assess their use as adjuvant in humans. Among the antigen delivery systems, orally given live virulence-attenuated bacteria seem to be promising candidates for the induction of immune responses. The phase I clinical trials on the use of live-attenuated *L. monocytogenes* as well as *S. typhimurium* in cancer immunotherapy showed that these bateria, and possibly other bacterial vectors as well, can become an alternative treatment in the future. There are still some limitations in their use as antigen carriers including differencies in protein post-translational modification between bacteria and eukaryotic cells, hindurances in folding of larger proteins or the limited number of proteins that can be expressed and secreted by bacteria [453]. The preexisting immunity might also affect the efficiency of the bacterial carrier. In addition, the use of live pathogen-based vectors raises concerns about safety in potentionally immunocompromised hosts and also the concerns about the release of genetically modified organisms into nature [442].

CONFLICT OF INTEREST

None declared.

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7. DISCUSSION

Part 1 (Publication 1, 4, 3)

7.1 CyaA as an antigen delivery tool and potent adjuvant

Over the past 20 years, various CyaA toxoids were used in mice without any added adjuvant to successfully deliver antigens and induce potent protective $CD8^+$ T cell-mediated immunity against viral infections, or as therapeutic vaccines against tumors (Adkins et al., 2012). This use was possible due to the capacity of the AC domain of the CyaA toxin to accommodate various epitope inserts without losing the capacity to penetrate cells (Sebo et al., 1995; Fayolle et al., 1996). Moreover, the CyaA toxoid was shown to deliver appropriate antigens and stimulate also $CD4^+$ T cells responses (Loucka et al., 2002; Schlecht et al., 2004). Currently, CyaA-based vaccines undergo clinical trials as immunotherapeutics for metastatic melanoma (Dadaglio et al., 2003). Unfortunately, CyaA-based vaccines failed in clnical trial phase II study for immunotherapy of HPV-induced cervical tumors (Preville et al., 2005; Mackova et al., 2006; www.genticel.com).

We focused here on analyzing the mechanism of penetration (important for antigen delivery) and adjuvanticity of the adenylate cyclase toxoid. Besides, we exploited the possibility to induce a protective immune response against influenza A viruses by using the CyaA toxoid mutant with inserted epitopes of HA2 subunit of hemagglutinin.

Publication 1

In our work we deciphered a novel intrinsic adjuvant activity of the adenylate cyclase toxoid (CyaA-AC^-). We show that a low concentration (300 ng/ml) of almost LPS-free (lower than 120 EU/mg) CyaA toxoid induces maturation of CD11b-expressing DC. By using BMDC from various knock-out mice, we excluded the dependence of this maturation process on several important TLR or inflammasome signaling pathways. The results allow to postulate that the maturation process of CyaA-AC^--treated DC is driven by the potassium efflux caused by the pore-forming activity of the toxoid and is regulated via JNK and p38 MAPKs. Moreover, CyaA-AC^--activated DC are able to stimulate T cell responses both *in vitro* and *in vivo*.

However, the toxoid induced incomplete phenotypic maturation of BMDC, as only some of the usual inflammatory molecules: KC (IL-8) and IL-6 cytokines, the chemokine LIX or the differentiation factor GCSF (granulocyte-colony stimulating factor) were produced. Interestingly, all of these molecules regulate predominantly the activities of neutrophils that are important mediators of innate immunity. We have not detected any significant production of the major pro-inflammatory cytokines such as TNF-α, IL-12 or IL-1β by toxoid-activated BMDC. This reflects the missing activation of BMDC by some of the TLR ligands, showing that these were absent in preparations of the CyaA toxoid. Importantly, this was confirmed by the capacity of CyaA-AC$^-$ to trigger CD80 and CD86 expression in BMDC from TLR (TLR 2, 4, 2/4, 9, TRIF, MyD88 and CD14) and inflammasome (IL-1R, P2X2, ASC and NLRP3) knock-out mice. These experiments clearly showed that if traces of TLR ligands were still present in the used toxoid preparations, their levels were too low to activate BMDC any importantly. While it was shown that CyaA mediated K^+ efflux contributed to activation of inflammasome and IL-1β production under condition of *B. pertussis* infection, there was no activation by the enzymatically inactive toxoid alone in the absence of BMDC priming through TLR signaling (Dunne et al., 2010). Similarly, there was no involvement of inflammasome signaling in the $CD8^+$ T cell stimulatory capacity of LPS-free CyaA-AC$^-$ *in vivo*, as recently shown by Dadaglio et al. (Dadaglio et al., 2014).

It has been reported previously that K^+ and Ca^{2+} ion signaling could induce maturation and migration of DC (Matzner et al., 2008; Shumilina et al., 2011). CyaA is capable of inducing K^+ efflux and Ca^{2+} influx, perturbing host cell ion homeostasis. We used a set of toxoid mutants with different abilities to influence the levels of these two ions in cells and found that the low pore-forming capacity of mutant CyaA-QR-AC$^-$ did not stimulate BMDC maturation and migration, despite inducing Ca^{2+} influx into cells to a similar extent as the normal toxoid CyaA-AC$^-$ (Bumba et al., 2010). On the contrary, the highly pore-forming toxoid CyaA-KK-AC$^-$ triggered very little Ca^{2+} influx (Fiser et al., 2012), while it still induced efficient BMDC maturation. Our results thus indicate, that it is the K^+ efflux caused by the pore-forming activity of the CyaA toxoid that is crucial for BMDC stimulation. We could, however, not rule out some role of Ca^{2+} signaling in this process either.

We further showed that CyaA-AC$^-$-mediated K^+ efflux activates p38 and JNK signaling pathways, which both are known to be important in numerous processes

underlying induction of adaptive immune responses such as DC maturation (Dong et al., 2002; Cargnello & Roux, 2011). Moreover, K^+ efflux from cells permeabilized by pore-forming toxins (PFTs) was recently shown to trigger specifically the phosphorylation of the kinases p38 (Kloft et al., 2009) and JNK (Kao et al., 2011). This appears to reflect a highly conserved mechanism of cellular response to PFT attack on cell membrane integrity, which is found in nematodes, insects and mammals (Porta et al., 2010; Huffman et al., 2004; Husmann et al., 2006; Bischof et al., 2008; Cancino-Rodezno et al., 2010; Gonzalez et al., 2011).

Similarly, we have observed that p38 and JNK activation were involved in upregulation of CCR7 expression and enhancement of the migratory capacity of BMDC towards CCL19 and CCL21 chemokines. This is in agreement with previous reports that p38 and JNK kinases participate in DC migration (Randolph et al., 2005; Iijima et al., 2005; Chladkova et al., 2011). Furthermore, p38 activity triggers synthesis and secretion of PGE2 (Norgauer et al., 2003) which might explain the observed enhancement of PGE2 production in BMDC after toxoid exposure.

We further show that a high dose of the intravenously administered CyaA-AC^- toxoid (25 µg) strongly enhances CD86 expression and upregulates CD80 and CD40 molecules on splenic $CD11c^+CD8^-CD11b^+$ DC *in vivo*. Intriguingly, at a dose of 25 µg per mice, the CyaA-QR-AC^- toxoid was still capable to rather efficiently trigger a lower but still significant expression of CD86 on splenic $CD11c^+CD8^+CD11b^+$ DC, despite its reduced cell-permeabilizing activity. We cannot exclude that at the high CyaA-QR-AC^- concentration its residual pore-forming activity may still promote enough K^+ efflux from DC to prime CD86 expression *in vivo*. Recently, Dadaglio et al. showed that after the intravenous application of even a higher dose of 50 µg of LPS-free CyaA-Tyr toxoid, the permeabilization-primed maturation of splenic DC was largely potentiated by signaling through the TLR4/TRIF pathway (Dadaglio et al., 2014). This signaling may result from a direct interaction/clustering of toxoid-CD11b/CD18 complexes with TLR4 receptors in the membrane of $CD11b^+$ DC. In keeping with these results, we showed that CyaA-AC^- at concentrations 0.1 - <1 µg/ml induced DC maturation solely via potassium efflux, whereas at concentrations ≥1 µg/ml, the CyaA-AC^- induced DC maturation through both the potassium efflux-triggered and the TLR4-dependent mechanisms, presumably operating in synergy. This is most likely potentiating the induction of antigen-specific T cell responses by CyaA toxoids. Indeed, the induction of antigen-specific $CD8^+$T cell response after

administration of CyaA-AC$^-$ carrying ovalbumin epitope SIINFEKL was shown to depend only partly on the TLR4/TRIF signaling triggered by the toxoid (Dadaglio et al., 2014). This is explained by our observation that the pore-forming activity of CyaA-AC$^-$ is necessary for toxoid-treated BMDC to induce T cell responses. We showed, indeed, that BMDC treated with low concentration of wild type CyaA-OVA-AC$^-$ (300 ng/ml), but not with the less pore-forming toxoid CyaA-OVA-QR-AC$^-$, were able to induce CD8$^+$ and CD4$^+$ T cell responses *in vitro* and expanded adoptively transferred CD8$^+$ T cells *in vivo*. Moreover, we observed a significantly higher expansion of adoptively transferred OVA specific CD8$^+$ and CD4$^+$ T cells after administration of 25 µg/ml of CyaA-OVA-AC$^-$ in comparison to administration of CyaA-OVA-QR-AC$^-$. This confirms the higher stimulatory activity of the pore-forming wild type toxoid towards antigen presenting cells *in vivo*. Based on these findings we proposed a concentration-dependent model of adjuvant activity of the immunotherapeutically relevant CyaA-AC$^-$ toxoid. It predicts that at low toxoid concentrations the cell permeabilizing activity provokes K$^+$-efflux-mediated p38 and JNK activation, triggering DC maturation, which is largely potentiated by TLR4/TRIF signaling upon high level of toxoid binding to cells.

The studies demonstrated that in line with the previously described ability to act as a good antigen delivery tool, the CyaA-AC$^-$ toxoid induces DC maturation, which is required for an efficient antigen-specific priming of naïve T cells (Fayolle et al., 1996; Adkins et al., 2012). Importantly, the toxoid was shown to induce a prominently Th1-polarized type of immune responses (Dadaglio et al., 2003; Ross et al., 2004; Mascarell et al., 2005), which is an observation potentially relevant also for the use of the toxoid as a new antigen and adjuvant in the next generation of acellular pertussis vaccines. Indeed, shifting of the predominantly Th2-polarized immune response to the currently used acellular pertussis vaccines (Ausiello et al., 1997; Ryan et al., 1998) towards induction of more Th1/Th17-polarized responses appears to be highly desirable in view of induction of longer-lasting and more efficient protective immunity against *B pertussis* infection (Ross et al., 2004; Mills et al., 2014). It is noteworthy that genetically fully detoxified CyaA-AC-Hly$^-$ (CyaA-QR-AC$^-$) toxoid (Osickova et al., 2010), protected by a patent portfolio, awaits evaluation as a novel aP vaccine component in the baboon weanling immunization model and *B. pertussis* challenge studies (Sebo et al., 2014).

Publication 4

The exact mechanism of AC domain translocation into cell cytosol across the lipid bilayer of cellular membrane remains poorly understood. CyaA translocation into cell cytosol depends upon a negative membrane potential (Otero et al.1995; Veneziano et al., 2013) and requires structural integrity of the acylated and calcium-loaded Hly moiety of the toxin (Bellalou et al., 1990; Rogel & Hanski, 1992; Hackett et al.1994; Masin et al., 2005). The four predicted transmembrane amphipathic α-helices (between residues 502 - 522 and 565 - 591) of the hydrophobic domain of CyaA contain pairs of negatively charged glutamate residues which have been found to be directly involved in the AC domain translocation across the target cell membrane (Osickova et al., 1999; Basler et al., 2007). However, membrane permeabillization by CyaA pores is not required for the process of CyaA translocation (Osickova e al., 2010).

Our study deciphered the contribution of the AC domain to the mechanism of its translocation across the cytoplasmic membrane by using a set of 18 CyaA mutants deleted in portions of their AC domain. We analyzed the capacity of all these CyaA deletion mutants to deliver ovalbumin antigen into the MHC class I presentation pathway. As a result we have shown that up to 371 amino acids in the AC domain are unnecessary for it to reach the cell cytosol. This was determined by the delivery of the MHC class I restricted T cell epitope of ovalbumin into DC followed by a successful stimulation of specific $CD8^+$ T cell response. It is thus plausible to suppose that the AC domain plays a rather passive role during the process of membrane penetration and does not contain any essential sequences needed for its penetration into cells. Moreover, the Hly moiety was able to deliver two large artificial polypeptides (146 and 203 amino acid residues long) into cell cytosol and to stimulate specific mouse and human CTL responses.

CyaA toxin penetrates into cell cytosol directly from the plasma membrane, without the need for endocytosis (Gordon et al., 1989; Bumba et al., 2010). This is quite a unique ability, as most other bacterial toxins having the ability to alter cAMP levels within the cell (e. g.: adenylate cyclase anthrax toxin of *Bacillus anthracis*, pertussis toxin of *Bordetella pertussis* or cholera toxin of *Vibrio cholerae*) are multicomponent A-B type toxins and require receptor-mediated endocytosis for penetration inside the cell. The fact that the AC domain penetration into the cytosol is a process independent of endocytosis was proven by the use of endocytosis

inhibitors. These inhibitors did not interfere with the translocation of the AC domain of CyaA as they did not prevent the elevation of the cytosolic cAMP or the delivery of $CD8^+$ T cell epitopes inserted into the AC domain into MHC class I presentation pathways of dendritic cells. (Bumba et al., 2010; Gordon et al., 1989; Guermonprez et al., 1999; Schlecht et al., 2004).

We showed that the delivery of the OVA epitope into cells was inhibited upon binding of the 3D1 antibody recognizing residues 373-399 of CyaA or by the deletion in the region beyond N-terminal 371 amino acids (construct B19). In line with these results, the segment linking the AC and Hly moiety might play a role in the translocation process (Subrini et al., 2013). Similarly, it was shown that the CyaA translocation was blocked by binding of 3D1 antibody or by deletion of residues 375-485 (Lee et al., 1999; Gray et al., 2001; Karst et al., 2012). To support this hypothesis, the recent results show that negatively charged residues within this linker segment control the size and the frequency of the formation of CyaA pores, while the positively charged residues seem to be engaged in the CyaA translocation process (Masin et al., 2016).

The self-adjuvanting capacity of the CyaA toxoid together with the possibility to replace the entire portion of the AC domain for the delivery of larger polyepitope antigens appear to be quite useful features of the CyaA toxoid, thus encouraging the design of a next generation of CyaA based vaccines.

Publication 3

As an application of the above discussed results, we tested CyaA-AC^- toxoids as a tool for delivery of an influenza vaccine.

Although influenza A viruses are being extensively studied, there is still no effective prevention against recurrent influenza infections. Influenza vaccines have to be updated each year according to the newly emerging strains. Current vaccines induce neutralizing antibodies targeted mainly against an immunodominant HA1 subunit of the surface glycoprotein hemagglutinin (HA). These specific antibodies then prevent influenza infection by blocking virus attachment to the cell surface. However, the problem is that this region of hemagglutinin is highly variable. Hence, some viruses can escape from immunity recognition. It is the reason why the development of a universal vaccine has not yet been successful. In order to avoid the

high rate of mutagenesis in influenza A viruses, current research is focused on defining of conserved antigens that could induce cross-protective antibodies and CTL responses against various seasonal influenza viruses (Stanekova & Vareckova, 2010).

While HA1 part of hemagglutinin is highly variable, HA2 part is well conserved. Furthermore, HA2 is known to be responsible for the fusion of viral and cell membranes in the endosomes, leading to the release of a ribonucleoprotein complex of the virus into the cytoplasm (Gerhard et al., 2006). There are many studies showing that HA-specific antibodies, targeting its stem region, can prevent this fusion step and inhibit viral replication both *in vitro* and *in vivo* (Stanekova & Vareckova, 2010). The HA2 antigen (residues 23-185), which we chose for our study, was from H3 subtype and contained several conserved T and B cell epitopes of influenza A virus (Saikh et al., 1995; Jackson et al., 1994). It was reported previously that HA2-based vaccine may induce a protective humoral response (Gocnik et al., 2008; Steel et al., 2010; Wang et al., 2010a,b; Bommakanti et al., 2010), but we showed for the first time that it may induce both $CD8^+$ and $CD4^+$ T cell responses, resulting presumably in enhanced immunity against influenza infection. Moreover, vaccination with CyaA-AC^--HA2 provided earlier clearance of influenza virus from mice lungs and protected mice against a lethal infection with homologous human influenza virus A/Miss H3N2 and importantly also against the heterologous highly pathogenic avian influenza virus A/Chick H7N1. Interestingly, this immunization proved to be protective against influenza infection up to $6xLD_{50}$ despite of the absence of any adjuvant.

Moreover, the induced HA2 specific antibodies were cross-reactive within the group 2 HA proteins H3, H4 and H7, but not with subtypes of H1. We further showed that the induced antibodies recognize the region $HA2_{76-130}$ exhibiting thus a similar specificity as the antibody 12D1 which was reported to be broadly neutralizing and cross-protective (Wang et al., 2010a, b). By the characterization of the antibody isotype composition we determined that CyaA-AC^--HA2 induced a mixed Th1 and Th2 immune response, while the vaccination with HA2 protein alone resulted rather in a Th2 polarization. This is in line with the previous reports of the CyaA toxoid inducing prominently Th1 polarized immune responses (Dadaglio et al., 2000; Mascarell et al., 2005). Moreover, we showed that the induced specific antibodies had the capacity to reduce viral replication. The important mechanism how HA2 antibodies might contribute to the milder course of the influenza infection is the

inhibition of virus fusion with host cell membranes (Vareckova et al., 2003; Gocnik et al., 2007; Wang et al., 2010a, b).

The induction of both $CD4^+$ and $CD8^+$ T cell immune responses is important during the influenza infection (McMurry et al., 2008). We observed an increase in the number of influenza virus specific IFN-γ secreting splenocytes in CyaA-AC^--HA2 immunized mice after restimulation with HA2-specific $CD4^+$ and $CD8^+$ T cell peptides. Additionally, it is plausible to suppose that also the cross-protective potential of the specific conserved region of HA2 (76-130) (Wang et al., 2010 a,b) might be enhanced by the CyaA mediated stimulation of T cell immunity targeting this part of HA2.

Influenza vaccines should be designed to elicit both antibody and T cell responses, as also T cell immunity plays an important role in clearance of the virus and contributes to the milder course of infection (Wang & Palese, 2009). Overall, our report was the first one to show a heterosubtypic protection against influenza A infection mediated by an HA2-based vaccine that can stimulate both arms of protective immunity without the need of adjuvant. This was previously possible only after vaccination with live attenuated influenza vaccines, which is not safe for everyone (Stropkovska et al., 2010). There is a possibility to design new CyaA-based vaccines that would be efficient against a broader spectrum of influenza A viruses. This might be achieved by a combination of selected conserved HA2 peptides from each subtype group. Recently, we have also shown that the AC domain of CyaA could be replaced by large polypeptides without affecting the antigen delivery capacity (Holubova et al., 2012). Such vaccines with a broadened protective efficacy would be important particularly to prevent possible worldwide influenza pandemic.

Part 2 (Publication 2, 5)

7.2 CyaA and its role in *Bordetella pertussis* infection

Despite massive vaccination world-wide, pertussis is re-emerging in the most developed countries that use the acellular pertussis vaccines. One of the key virulence factor of this bacteria is the adenylate cyclase toxin - CyaA, a swift saboteur of host cell functions (Vojtova et al., 2006). Its role in pertussis has been studied,

however more details are needed to understand its specific mechanism of action. Hence, these studies were focused on the deciphering the mechanisms of how the CyaA toxin manipulates dendritic cells and what is the effect of the pore-forming activity of the CyaA toxin in the course of infection.

Publication 2

Upon the penetration into cell cytosol, the CyaA toxin generates a rapid elevation of concentration of cAMP, an important second messenger. This interferes with bactericidal functions of phagocytes by inhibition of superoxide production, chemotaxis and phagocytosis (Confer & Eaton, 1982; Pearson et al., 1987; Vojtova et al., 2006). Moreover, it was shown that CyaA can promote apoptosis (Khelef et al., 1993) or necrosis (Basler et al., 2006). We reported that the CyaA action also inhibits macropinocytic uptake (Kamanova et al., 2008). Recently, Cerny et al. showed that the Cya-mediated PKA activation leads to the activation of the tyrosine phosphatase SHP-1, which inhibited NO production by macrophages (Cerny et al., 2015). Furthermore, SHP-1 activation resulted in the stabilization of the proapoptotic protein BimEL, Bax activation and thus induced macrophage apoptosis (Ahmad et al., 2016).

Our study aimed on a deeper characterization of the immunomodulatory properties of the CyaA toxin acting on TLR-stimulated murine and human DC (BMDC and MDDC). Importantly, throughout our work we used as low physiological concentration of the CyaA toxin as 10 ng/ml (Eby et al., 2013). In order to decipher the role of CyaA action during pertussis infection we mimicked an infection-like environment by using LPS that is a very potent TLR-activator of DC during infection. LPS from *E. coli* may differ from *B. pertussis* LOS in the ability to interact with the TLR4-MD-2-CD14 complexes both in mice and humans (Hajjar et al., 2002; Marr et al., 2010).

It was previously shown that the CyaA toxin has the capacity to modulate TLR-induced maturation of DC (Ross et al., 2004; Boyd et al., 2005; Spensieri et al., 2006). In accordance with these studies, we observed that cAMP signaling of CyaA increased IL-10 secretion and decreased IL-12p70 secretion and CD40 expression in TLR-activated mouse and human DC. In addition, we showed that CyaA enhanced LPS-induced cell detachment and chemotactic migration of DC. Moreover, CyaA decreased the capacity of LPS activated DC to stimulate proliferation of antigen-

specific $CD4^+$ and $CD8^+$ T cells *in vitro* and *in vivo*. Furthermore such DC decreased the induction of IFN-γ producing $CD8^+$ T cells, while it enhanced the induction of antigen-specific IL-17 and IL-10 producing $CD8^+$ T cells and of $CD4^+CD25^+Foxp3^+$ T regulatory cells.

We showed that the CyaA-mediated cAMP signaling enhanced TLR-induced dissolution of cell adhesive contacts and promoted migration of DC towards the lymph node homing chemokines CCL19 and CCL21. However, we did not observe any correlation between the amount of CCR7 on the DC cell surface after 24 h and the enhanced chemotactic migration of CyaA and LPS-treated DC, suggesting that the LPS-induced CCR7 expression was sufficient for enhanced migration along the cytokine gradient. Indeed, as documented by the xCelligence measurements, DC treated with CyaA and LPS exhibited a faster dissolution of adhesive contacts, possibly facilitating migration. It has been shown that actin and myosin inhibitors reduced the speed of migration but not the directed motion (Ricart et al., 2011) and the integrin-ligand binding properties of cells similarly affect the migration speed (Palecek et al., 1997). It remains to be established, however, to which extent the capacity of CyaA to increase migration might play a role during *B. pertussis* infection *in vivo*, because wild-type *B. pertussis* was shown to inhibit migration of MDDC towards CCL21 *in vitro* in dependence on the presence of active PT (Fedele et al., 2011).

Moreover, as further shown by us, LPS stimulated DC after exposure to CyaA exhibit a decreased capacity to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells. It was demonstrated previously that T cells isolated from lungs of mice infected by *B. pertussis* were impaired in the capacity to respond to *Bordetella* antigens (McGuirk et al., 1998). Additionally, CyaA caused *B. pertussis* infected human monocytes mediated inhibition of antigen-dependent $CD4^+$ T cell proliferation in response to tetanus toxoid (Boschwitz et al., 1997). We have ruled out several possible mechanisms how CyaA could modulate these immune responses: induction of DC death, decrease in MHC II or costimulatory molecule expression, production of inhibitory soluble factors, modulation of cytokine secretion, inhibition of ovalbumin uptake or processing or decrease in viability of T cells. Altogether, our results suggest, that CyaA via its cAMP signaling interferes with the MHC class II antigen presentation pathway downstream to antigen uptake and degradation. One possible explanation could be that CyaA causes perturbation of vesicular sorting and

trafficking of epitope-loaded MHC II molecules from endosomes to the cell surface as we previously reported that CyaA induced massive actin cytoskeleton rearrangements and membrane ruffling in CD11b-expressing myeloid cells (Kamanova et al., 2008). Recently it has been reported that cAMP signaling shapes DC to a tolerogenic phenotype and subverts their T cell stimulatory capacity (Challier et al., 2013). Moreover, also cholera toxin of *V. cholera* and heat-labile enterotoxin of *E. coli* trigger a cAMP signaling pathway which by a yet not very well known mechanism interferes with the proper presentation of antigens by macrophages or B cell lymphoma (Matousek et al., 1996; Matousek et al., 1998; Tanaka et al., 1999).

We further showed that the CyaA treatment of DC decreased their ability to induce IFN-γ-secreting $CD8^+$ T cells, while it promoted antigen-specific IL-10 and IL-17-producing $CD8^+$T cells. This is in line with previously published data that cAMP accounts also for the development of Th17 cells (Anderson & Gonzalez-Rey, 2010). These together with Th1 cells were shown to be important for clearance of *B. pertussis* from the respiratory tract of infected mice (Dunne et al., 2010). Although $CD8^+$ T cells do not seem to be necessary for the establishment of the protective immunity against *Bordetella* in mice (Leef et al., 2000; Mills et al., 1993), IFN-γ producing $CD8^+$ T cells were detected in *B. pertussis* infant and young adult patients and in vaccinated children (Dirix et al., 2012; Rieber et al., 2011). Besides, they might also contribute to the defense against secondary infections. Another possible role for them is in the defense against infected human macrophages, where *B. pertussis* may survive and even grow for some time (Lamberti et al., 2010).

It has been reported that $CD25^-Foxp3^+$ T regulatory cells are the predominant suppressive subtype in the lungs of mice infected by *B. pertussis* (Coleman et al., 2012). Furthermore, CyaA was previously shown to induce IL-10-producing T regulatory cells (Tr1) through manipulation of DC (Ross et al., 2004). Recently it was shown that CyaA may stimulate IL-10 production by inhibitory phosphorylation of SIK family of kinases, probably mediated by PKA, via the dephosphorylation and nuclear translocation of transcriptional coactivator CRTC3/TORC3 (Novak et al., in preparation). Moreover, the LPS-free CyaA stimulated DC accounted for expansion of $CD4^+CD25^+Foxp3^+$ T regulatory cells *in vitro*. However, it needs to be determined what role has this Treg population in the course of *B. pertussis* infection, whether such T cells may subvert the bacterial clearance or limit the immune-mediated pathology (Higgs et al., 2012).

As the key function of DC is to induce the adaptive immune response, the results of our study support the hypothesis that the CyaA toxin prevents the onset of the effective adaptive immune response during *B. pertussis* infection. Although CyaA promotes migration of DC, it interferes with their capacity to stimulate proper T cell responses by manipulating their TLR-induced maturation process. Such manipulated DC are then unable to stimulate adequate T cell responses not only by inhibition of their induction, but also by shaping the resulting T cell profile towards more tolerogenic type and thereby delay the clearance of bacteria and prolong the persistence of *B. pertussis* infection.

Publication 5

We constructed a unique *B. pertussis* mutant that produces a cell-invasive but non-hemolytic variant of the CyaA toxin (AC^+Hly^-). We showed that the pore-forming activity of the secreted CyaA is not required for the capacity of *B. pertussis* to colonize mouse lungs. However, it significantly contributes to the virulence and lethality of *B. pertussis* infection in the mouse respiratory challenge model.

Intriguingly, the cell-permeabilizing activity of CyaA accounted for an important pathology provoked in mouse lungs by the hemolytic *B. pertussis* bacteria already at inoculation doses as low as 10^5 CFU. Significantly milder lung pathology was then observed at comparable levels of lung colonization by the non-hemolytic AC^+Hly^- bacteria. The AC^+Hly^- mutant was then mostly found attached to the respiratory epithelium brush border in the bronchial lumen, while the parental hemolytic AC^+Hly^+ *B. pertussis* bacteria penetrated the epithelial layer and infiltrated the parenchyma to form pneumonic foci. The selective impairment of the hemolytic (pore-forming) activity thus significantly reduced the invasiveness and virulence of *B. pertussis* infection, where death of mice occurred about a week later and at an order of magnitude higher inoculation dose, than upon infection with the wild-type (AC^+Hly^+) bacteria. This suggests that in the mouse model of respiratory challenge, much of the virulence and lethality of *B. pertussis* infections is likely due to a 'suicidal' hyperactivation of innate host defense and exacerbated lung inflammation caused at least in part by the cell-permeabilizing activity of the CyaA toxin.

Neutrophil recruitment and inflammation of infected tissue would, indeed, possibly be potentiated by the synergy of the cell-permeabilizing and cell-invasive AC enzyme activities of CyaA. These toxin activities may trigger inflammation by at least

three known mechanisms. CyaA was shown to elicit production of IL-6 in tracheal epithelial cells (Bassinet et al., 2000; Gueirard et al., 1998; Khelef et al., 1994), where IL-6 is known to activate cytotoxic action of neutrophils at the site of infection. Secondly, cAMP signaling of CyaA was shown to induce COX-2 expression in macrophages, which would potentially yield release of prostaglandins and chemo-attraction of neutrophils (Perkins et al., 2007). The third mechanism would involve the contribution of CyaA-mediated potassium efflux to NALP3 inflammasome assembly in LPS-primed dendritic cells, which yields caspase-1 activation and pro-inflammatory IL-1β secretion (Dunne et al., 2010). IL-1β then can trigger neutrophil chemokine production by non-immune cells (Miller et al., 2006) and potentiate neutrophil infiltration into lung parenchyma, thereby provoking inflammatory damage of the infected lungs.

The colonization defect of the $AC^{-}Hly^{+}$ mutant observed by us was much less pronounced than what was reported earlier for infections with about one and a half order of magnitude higher challenge doses ($10^{6.5}$ – 10^{7} CFU) of the $AC^{-}Hly^{+}$ mutant or with the CyaA-deficient bacteria (Khelef et al., 1992). It is possible that at such high inoculation doses the excessive harnessing of host response and neutrophil infiltration would allow fast elimination of the $AC^{-}Hly^{+}$ bacteria that cannot neutralize phagocytes by production of cytosolic cAMP. In contrast to the parental strain, however, the $AC^{-}Hly^{+}$ bacteria inoculated at only 10^{5} CFU were not completely cleared from mouse lungs for up to 30 days and persisted at a low but detectable level of about 10^{3} CFU (data not shown), suggesting that the $AC^{-}Hly^{+}$ mutant might persist much longer in lung alveoli or inside host cells because of being less cytotoxic to epithelial or phagocytic cells.

The non-hemolytic $AC^{+}Hly^{-}$ mutant colonized mouse lungs as efficiently as the parental $AC^{+}Hly^{+}$ *B. pertussis* strain. This goes well with the previous reports on the capacity of CyaA to disarm neutrophils, promote apoptosis of alveolar macrophages and subvert *in vitro* the TLR ligand-induced maturation and cytokine profiles of dendritic cells, respectively (Bagley et al., 2002; Boschwitz et al., 1997; Fedele et al., 2010; Hickey, et al., 2008; Njamkepo et al., 2000; Ross et al., 2004; Spensieri et al., 2006). We confirmed that *B. pertussis*-produced CyaA accounted for suppression of IL-12 and TNF-α secretion and for enhanced release of immunosuppressive IL-10 by mouse DCs in contact with live *B. pertussis* bacteria. The CyaA also interfered with the presentation of the OVA antigen on DCs exposed to live *B. pertussis* bacteria and

reduced the level of induction of antigen-specific IFNγ secretion by T cells, while enhanced antigen-specific secretion of IL-17A and IL-10 by OVA-specific $CD8^+$ and $CD4^+$ T lymphocytes. Moreover, the enzymatic activity of *B. pertussis*-secreted CyaA promoted the expansion of antigen-specific $Foxp3^+CD25^+CD4^+$ T regulatory cells by the DCs *in vitro.* The same observation was, indeed, made recently with purified recombinant CyaA, which at a concentration of only 10 ng/ml was able to skew the antigen-presenting capacities of LPS-activated DCs towards a tolerogenic phenotype (Adkins et al., 2014). Since similar CyaA concentrations were recently found in nasal aspirates of infants with pertussis and in nasopharyngeal washes of *B. pertussis*-infected baboons (Eby et al., 2013), it is plausible to assume that the local concentrations of CyaA at infected epithelial surfaces might be even higher. CyaA could thus play a major role in suppression of adaptive T cell immune response during natural *B. pertussis* infections. It is further possible to hypothesize that the cAMP-elevating activity of secreted CyaA may be contributing to the previously observed expansion of regulatory T cells in *B. pertussis* infected tissues *in vivo* (Higgs et al., 2012). Indeed, the use of the AC^+Hly^- mutant allowed us to conclusively show that the pore-forming activity of CyaA did not contribute to the cAMP-triggered tolerogenic shaping of the phenotype of DCs exposed to TLR ligands produced by live *B. pertussis in vitro*.

Intriguingly, however, the hemolytic activity of CyaA played a role in increased macrophage, $Ly6C^{high}$ monocyte, conventional and plasmacytoid DC arrival into mouse lungs infected by the parental AC^+Hly^+ *B. pertussis*. At the same time, the cell-permeabilizing activity synergized with the cAMP signaling activity of the CyaA toxin in bringing about a significant reduction of the mean level of MHC II molecule expression on myeloid cells in the infected lungs *in vivo*. While the mechanism by which this reduction of MHC II levels occurs remains enigmatic, it was observed here for several types of antigen presenting cells, such as macrophages, monocytes, pDCs or cDCs. It is thus likely to have non-negligible repercussions on the efficacy of $CD4^+$ T cell immune response induction during *B. pertussis* infection *in situ,* at the site of infection. Subversion of maturation and of antigen presenting capacities of intraepithelial DCs due to combined cAMP-elevating and cell-permeabilizing action of CyaA would plausibly be expected to interfere with adaptive responses of $CD4^+$ T cell help-depending B lymphocytes. Delaying and restricting the efficacy of antibody response induction and limiting development of B and T cell immune memory to *B.*

pertussis antigens, may hence represent a second wave activity of CyaA, following on the incapacitation of the frontline defense exerted at the site of infection by the CR3-expressing neutrophils and macrophages, which are particularly sensitive to CyaA toxin action.

8. CONCLUSIONS

Mechanism of CyaA-AC$^-$ adjuvanticity (Publication 1)

- We show that non-enzymatic but pore-forming CyaA-AC$^-$ toxoid at low concentration of 300 ng/ml induces the maturation of CD11b-expressing dendritic cells by a TLR- and inflammasome-unrelated mechanism. This depends on the cell-permeabilizing capacity of CyaA-AC$^-$ and involves the activation of the JNK and p38 protein kinases.
- Upon toxoid treatment, bone marrow-derived dendritic cells (BMDC) increased the expression of co-stimulatory molecules, production of IL-6, KC, LIX, GCSF, and PGE2 and exhibited increased chemotactic migration
- Moreover, we show that CyaA-AC$^-$-mediated K^+ efflux induces the maturation of splenic DC and their capacity to expand antigen-specific $CD4^+$ and $CD8^+$ T cells *in vivo*
- It is therefore plausible to propose that it is the capacity of the CyaA-AC$^-$ toxoid to trigger the maturation and migration of DC that accounts for its adjuvant capacity *in vivo*.

CyaA modulates TLR-induced maturation and T cell stimulatory capacity of DC (Publication 2)

- We examined the ability of close-to-physiological concentration of the CyaA toxin to modulate LPS-induced maturation and T cell stimulatory capacity of mice and human DC through activation of cAMP signaling pathway
- CyaA supports LPS-induced cell detachment and migration towards the lymph node homing chemokines CCL19 and CCL20
- We showed that CyaA decreased the capacity of TLR-activated DC to present soluble antigens to $CD4^+$ T cells and increased their capacity to promote expansion of $CD4^+CD25^+Foxp3^+$ T regulatory cells *in vitro*
- Moreover, CyaA also decreased the capacity of LPS-stimulated DC to induce $CD8^+$ T cell proliferation. In addition, it resulted in the reduced induction of IFN-γ production while it enhanced the induction of IL-17 and IL-10 production by $CD8^+$ T cells.

Protection against influenza A induced by CyaA-AC$^-$-HA2 (**Publication 3**)

- The prepared CyaA-AC$^-$-HA2 toxoid induces HA2 specific T and B cell responses in BALB/c mice. The presence of specific antibodies was detected by ELISA in the sera of immunized mice. The isotype composition of the specific antibodies suggested an induction of mixed Th1 and Th2 polarized immune response.
- We further show that HA2-specific antibodies induced by CyaA- AC$^-$-HA2 were cross-reactive with influenza A viruses of the H3, H4 and H7 subtypes. In addition, they were shown to reduce the virus replication.
- CyaA- AC$^-$-HA2 immunization accelerated virus elimination from mice infected with homologous H3 and heterologous H7 influenza viruses and protected them against a lethal dose of these viruses.

Delivery of truncated AC domain across the cytoplasmic membrane of APCs (Publication 4)

- Deletion mapping results showed that the first 371 amino acids of the AC domain are dispensable for the capacity of CyaA to translocate its N-terminal portion across the cytoplasmic membrane of dendritic cells. This was determined by delivery of an OVA epitope into DC for *in vitro* stimulation of OVA-specific CD8$^+$ T cells.
- Toxoids in which the first 371 residues of the AC domain were replaced by long heterologous polyepitope were still capable to induce effective antigen-specific CD8$^+$ CTL responses *in vivo* in mice and *ex vivo* in human peripheral blood mononuclear cell cultures.
- The results suggest that the AC domain participates in the membrane penetration only as a passive passenger. Importantly, its ability to deliver large heterologous polypeptides across the cytoplasmic membrane of antigen presenting cells paves the way for the construction of a new generation of CyaA-based vaccines.

The role of the pore-forming activity of CyaA in Bordetella infection (Publication 5)

- Hemolytic (pore-forming) activity of CyaA is not required for immunomodulatory shaping of dendritic cell phenotype by *B. pertussis*
- Hemolytic activity of CyaA is not required for mouse lung colonization by *B. pertussis,* however, it contributes to exacerbation of inflammatory damage of mouse lungs in the course of infection
- Moreover, the pore-forming activity of CyaA accounts for recruitment of myeloid cells into infected lungs
- The pore-forming activity synergized with the cAMP-elevating activity in downregulation of MHC II molecule expression levels on infiltrated myeloid cells

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