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FACULTY OF SCIENCE

DEPARTMENT OF CELL BIOLOGY

DIPLOMA THESIS

Immunomodulation of dendritic cells by adenylate cyclase toxin from *Bordetella pertussis*

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I hereby declare that this Diploma Thesis is based on my own work under the supervision of Mgr. Ireny Adkins, Ph.D, and that all the resources employed as well as co-authors are indicated.

Hana Jáňová .....

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Abstrakt

Bordetella pertusis je gram-negativní bakterie, která způsobuje onemocnění černý kašel. Mezi hlavní faktory virulence této bakterie se řadí adenylát cyklázový toxin (CyaA), který se uplatňuje v kolonizaci hostitele. Toxin se váže s vysokou afinitou na myeloidní fagocyty nesoucí na svém povrchu CD11b/CD18 integrin, proniká do jejich cytosolu a negativně ovlivňuje jejich schopnost fagocytosy, chemotaxe a oxidativního vzplanutí. Dále také CyaA ovlivňuje spektrum sekretovaných cytokinů u dendritických buněk (DC) stimulovaných LPS a moduluje jejich maturaci tím, že působí na expresi ko-stimulačních molekul. V této práci jsme se zabývali vlivem CyaA toxinu na schopnost myších DC z kostní dřeně presentovat ovalbuminové epitopy vnesené CyaA-AC$^-$ toxoidem a aktivovat CD4$^+$ a CD8$^+$ T-buňky. Dále jsme se snažili zjistit, zda DC ovlivněné působením CyaA snižují CD4$^+$ T buněčnou odpověď v důsledku sníženého antigenního příjmu nebo jeho zpracování pro presentaci na MHC molekulách II. třídy. CyaA také snižuje CD8$^+$ T-buněčné odpovědi, proto jsme se zaměřili na vliv toxinu na proteolytickou aktivitu proteasomu a na možné ovlivnění prezentace peptidů na MHC molekulách I. třídy.

Zjistili jsme, že zvýšená hladina cAMP v DC snižuje presentaci ovalbuminových epitopů vnesených CyaA-AC$^-$ toxoidem na MHC molekulách I. a II. třídy. Tím dochází ke snížení stimulace CD8$^+$ a CD4$^+$ T buněk. Dále jsme zjistili, že CyaA neovlivňuje příjem antigenů pomocí receptorem zprostředkované endocytosy, ale inhibuje makropinocytosu u DC. Nicméně, CyaA nevykazuje inhibiční efekty na zpracování ovalbuminu pro vazbu na MHC molekuly II. třídy, ani na proteolytické funkce proteasomu produkujícího peptidy pro vazbu na MHC molekuly I. třídy. Zdá se tedy pravděpodobné, že schopnost CyaA snižovat T-buněčnou odpověď v našem systému není důsledkem ovlivnění příjmu a zpracování antigenů v DC, ale je pravděpodobně hlavně důsledkem snížené exprese ko-stimulačních molekul a produkci inhibičních cytokinů, jako je například IL-10.

Klíčová slova: adenylát cyklázový toxin (CyaA); cAMP; dendritické buňky; T buňky; antigenní presentace; zpracování a příjem antigenů; *Bordetella pertussis*

Abstract

Adenylate cyclase toxin (CyaA) produced by the causative agent of whooping cough *Bordetella pertussis*, is a key virulence factor important for colonization of the host. CyaA targets preferentially myeloid phagocytes expressing CD11b/CD18 integrin. By elevating cytosolic cAMP in the host cells, CyaA interferes with their phagocytic, chemotactic and oxidative burst capacities. Furthermore, CyaA modulates the secretion of cytokines and the maturation state in LPS-stimulated dendritic cells (DC) by affecting the expression of co-stimulatory molecules. In this study, we investigated the effects of CyaA on the capacity of murine bone-marrow DC to prime $CD4^+$ and $CD8^+$ T cells in response to ovalbumin epitopes delivered by the CyaA-AC^- toxoid, as a model antigen. Further, we examined the possible impact of CyaA on the antigen uptake and processing for MHC class I and II-restricted presentation by DC, as we previously observed a decreased T cell stimulatory capacity of CyaA-treated DC in response to soluble ovalbumin.

We found out that the high levels of cAMP generated by CyaA in LPS-stimulated DC account for the decreased presentation of ovalbumin epitopes carried by CyaA-AC^- toxoid on MHC class I and II molecules, thereby impairing the $CD8^+$ and $CD4^+$ T cell responses. Whereas CyaA did not influence the antigen uptake via receptor-mediated endocytosis, it notably decreased the macropinocytosis in DC. However, CyaA did not affect antigen processing neither for MHC class I nor for MHC class II presentation in DC. Therefore it seems possible, that in our model system the ability of CyaA to decrease T cell-stimulatory capacity of DC is mainly due to the inhibition of the expression of co-stimulatory molecules and the production of immunomodulatory cytokines like IL-10.

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

Content

1. ABBREVIATIONS

8-Br-cAMP	8-Bromoadenosine 3',5'-cyclic monophosphate
AC	Adenylate cyclase
ADP	Adenosine diphosphate
Ag	Antigen
AMC	7-amino-4-methylcoumarin
APC	Antigen presenting cells
APS	Amonium persulfate
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BMDC	Bone morrow-derived dendritic cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CLIP	Class II-associated invariant-chain peptide
CRPG	Chlor phenol red-β-D-galactopyranoside monosodium salt,
cSMAC	Central supramolelular activation complex
CT	Cholera toxin
CTL	Cytotoxic T lymphocytes
CyaA	Adenylate cyclase toxin (=ACT)
cyaA	Gene encoding adenylate cyclase toxin
cyaC	Gene encoding acyltransferase
db-cAMP	Dibutyryl cyclic adenosine monophosphate
DC	Dendritic cells
DEAE	Dietylaminoethyl
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
DTT	Dithiotreitol
E. coli	Escherichia coli
EBV	Epstein-Barr virus
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EPAC	Guanine nucleotide-exchange protein directly activated by cAMP
ER	Endoplasmatic reticulum
ERK	Extracellular-signal-regulated kinases
FACS	Flow cytometry
FCS	Fetal calf serum
FHA	Fillamentous hemagglutinin
FITC	Fluoroscein isothiocyanate
GA	Golgi apparatus
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine-5'-triphosphate
HEL	Hen egg lysosome
HEPES	2-hydroxyethyl-1-piperazineethanesulfonic acid
HLA-DR	Human leukocyte antigens DR
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IKDC	Interferon-producing killer DC
IL	Interleukin
IPTG	Isopropyl-β-D-thiagalactopyranosid
kDa	kiloDallton
KLH	Keyhole limpet hemocyanin
LacZ	β-galactosidase
lacZ	Gene for β-galactosidase
LB medium	Luria Bertani medium
LPS	Lipopolysaccharide
LT	Enterotoxin
MAPK	Mitogen activated protein kinases
MDDC	Monocyte derived dendritic cells
MFI	Mean fluorescence intensity
MHC	Major histocompability complex
MIIC	MHC class II-rich compartments
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NKT cells	Natural killer T cells

OPD	o-Phenylenediamine
OVA	Ovalbumin
P_a	Acellular pertussis vaccines
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraphenyl aldehyde
PGE2	Prostaglandin E2
pSMAC	Peripheral supramolecular activation complex
PT	Pertussis toxin
P_w	Whole perussis vaccines
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Electrophoresis in polyacrylamid gel in the presence of SDS
SPI	Salmonella pathogenicity island
TCM	Central memory T cells
TCR	T cell receptor
TCT	Tracheal cytotoxin
T_{EM}	Effector memory T cells
TEMED	N,N,N', N'-tetramethylendiamine
Th	T helper cells
TMRE	Tetramethylrhodamine, ethyl ester, perchlorate
TNF	Tumour necrosis factor
Treg	T regulatory cells
USF	Upstream stimulatory factor
λ-DNA	Bacteriophage lambda DNA

2. OBJECTIVES AND GOALS

Bordetella pertussis is the causative agent of whooping cough, a respiratory disease that affects millions of children throughout the world each year. Despite high vaccination rates, 254 thousands of people died due to Pertussis according to World Health Organisation (WHO [http://www.who.int]), in year 2004 only, which makes up 0.4 % of all deaths registered by WHO in that year. *B. pertussis* produces a number of potent virulence factors, adhesins and toxins, among which is also adenylate cyclase toxin (CyaA, ACT or AC-Hly). CyaA plays an important role in early stages of respiratory tract colonization and in the establishment of the infection (Goodwin & Weiss, 1990). Upon *B. pertussis* colonization, CyaA interacts with tracheal epithelial cells and induces IL-6 production ((Bassinet *et al.*, 2004). Secreted CyaA binds to the cells of immune system expressing $\alpha_M\beta_2$ integrin CD11b/CD18 (CR3 or Mac-1) (Guermonprez *et al.*, 2001) such as neutrophils, macrophages, natural killer cells and dendritic cells that are attracted to the site of infection (Guermonprez et al., 2001, El-Azami-El-Idrissi *et al.*, 2003). CyaA not only elevates cAMP level, an important second messenger and affects various cellular signalling cascades leading to the inhibition of phagocytosis, oxidative burst, cytokine production and chemotaxis (Boyd *et al.*, 2005, Friedman *et al.*, 1987) but also perturbs ion cell homeostasis by forming cation-selective pores (Vojtova *et al.*, 2006). In dendritic cells (DC) CyaA modulates cytokine secretion and maturation (Boyd et al., 2005, Ross *et al.*, 2004, Skinner *et al.*, 2004, Spensieri *et al.*, 2006, Fedele *et al.*, 2010). However, little is known about the effects of CyaA on the capacity of DC to stimulate T cell adaptive responses, especially CyaA effects on the antigen uptake and processing in DC. In our laboratory we observed that CyaA inhibits the ability of DC to stimulate $CD8^+$ and $CD4^+$ T cells (Adkins et al., prepared for submission) in response to soluble ovalbumin. CyaA was also found to inhibit macropinocytosis in macrophages (Kamanova *et al.*, 2008). Therefore the investigation of CyaA effects on the T cell-stimulatory capacity of DC might help us in coming closer to understanding how *B. pertussis* might manipulate host immune cells during the infection.

Aims of the Diploma Thesis

1) To investigate whether CyaA influences the capacity of bone marrow-derived DC to stimulate $CD8^+$ and $CD4^+$ T cells in response to ovalbumin epitopes carried by CyaA-AC^- toxoid (CyaA-OVA-AC^-), as a different model antigen similarly to a soluble ovalbumin.

2) To investigate whether CyaA affects the antigen uptake in DC as a possible reason for the impairment of $CD4^+$ T cell response.

3) To investigate whether CyaA affects the antigen processing for MHC class I and II presentation in DC as a possible reason for their decreased $CD4^+$ as well as $CD8^+$ T cell-stimulatory capacity.

The data obtained within this diploma thesis are prepared for publication:

Bordetella adenylate cyclase toxin decreases $CD4^+$ and $CD8^+$ T cell expansion via modulation of dendritic cells
Irena Adkins, Jana Kamanova, Jakub Tomala, Hana Janova, Jiri Masin, Marek Kovar and Peter Sebo

3. INTRODUCTION

3.1 *Bordetella pertussis*

The genus *Bordetella* subdivides into several species, from which only four*, B. pertussis, B. bronchiseptica, B. parapertussis*, and *B. holmesii* are associated with respiratory infections in humans and other mammals. *B. pertussis* is a causative agent of whooping cough, *B. bronchiseptica* infects a wide range of species, human-adapted *B. parapertussis* causes a milder pertussis-like disease, and most recently discovered *B. holmesii* is associated with human respiratory tract infection.

Bordetella pertussis, an agent of whooping cough, is a strictly human pathogen, although it can infect other mammals as well. Bacteria colonize the mucosa of the upper respiratory tract and synthesize a variety of virulence factors, including adhesins and toxins (Mattoo & Cherry, 2005). The pathogenic factors of Bordetella are depicted in Fig 1. Beside lipopolysaccharide (LPS) which differs in structure from that of *E. coli* (Caroff *et al.*, 2001), the most important pathogenic factors are pertussis toxin (PT), adenylate cyclase toxin (CyaA), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), type III secretion system (TTSS) and adhesins, such as filamentous hemagglutinin (FHA) (Mattoo & Cherry, 2005). Noteworthy virulence factors include also fimbriae (FIM), which are filamentous cell surface structures that are required for persistent tracheal colonization; pertactin (PRN), a surface protein that mediates eukaryotic cell binding in vitro; and BrkA, a putative adhesin conferring serum resistance and protection against antimicrobial peptides (Locht *et al.*, 2001).

Pertussis toxin (PT), which is exclusively secreted by *Bordetella pertussis*, has been associated with most of systematic clinical symptoms caused by *B. pertussis*, such as leukocytosis, insulinemia/hypoglycaemia and histamine sensitivity (Carbonetti *et al.*, 2007). In mouse intranasal infection model, it has been found that PT is important at the very beginning of the infection targeting airway macrophages and neutrophils (Carbonetti *et al.*, 2003). PT is a complex multisubunit toxin with an AB5 configuration (one active subunit plus five binding subunits. The enzymatic activity of PT resides in the A subunit, while the B oligomer binds to eukaryotic cell membranes and dramatically increases the efficiency with which the S1 subunit gains entry into host cells. PT ADP-ribosylates several heterotrimeric G proteins in mammalian cell, thus stimulating the adenylate cyclase to generate cAMP. The elevated levels of cAMP account for the pathogenic effects of PT on the host cells (Carbonetti, 2010). Detoxified PT is used as a primary component of pertussis vaccines (Carbonetti et al., 2007) acting as a potent mucosal adjuvant.

Dermonecrotic toxin (DNT) is a secreted A-B toxin, which has also been called heat-labile toxin, because of its complete inactivation after 60 min at 56 °C. Once the toxin has reached the cytoplasm of the target cells its enzymatic domain activates small GTP-binding protein Rho leading to profound alterations in the actin cytoskeleton. Even though the role of DNT in the pathogenesis has not been dissected yet, it is a very potent toxin, as an intravenous injection of very low doses of DNT is lethal for mice (Mattoo & Cherry, 2005).

Tracheal cytotoxin (TCT) is a disaccharide-tetrapetide monomer of peptidoglycan which is in relatively high levels released by *B. pertussis*. In association with LPS, TCT damages the ciliated airway epithelial cells by triggering of IL-1α and nitric oxide production. It also causes mitochondrial bloating, disruption of tight junctions and cell blebbing (Carbonetti et al., 2007, Mattoo & Cherry, 2005).

Adenylate cyclase toxin (CyaA, ACT) is involved in the early stages of the respiratory tract colonization by *B. pertussis* (Vojtova et al., 2006)). CyaA translocates into cells and upon binding of calmodulin catalyzes unregulated conversion of ATP to cAMP primarily affecting functions of immune cells. This toxin is in more details described in chapter 3.2.

Filamentous hemagglutinnin, a major *B. pertussis* adhesin, mediates the attachment to the variety of cells and extracellular structures in the respiratory epithelium, such as epithelial cells and macrophages. According to these characteristics, it is required for tracheal colonization. As it is highly immunogenic, it is also included as a primary component in acellular pertussis vaccines (Mattoo & Cherry, 2005, Locht et al., 2001).

Type III secretion system (TTSS) has been identified in *Bordetella* subspecies and recently a functional TTSS was also described in *B. pertussis* (Fennelly *et al.*, 2008). TTSS serves as needle-like injection apparatus that allows gram-negative bacteria translocate effector proteins directly into the plasma membrane or cytoplasm. These effector proteins then convert normal host cell-signalling cascades and other processes to promote the pathogenic strategies of the bacteria (Mattoo & Cherry, 2005). TTSS from *B. bronchiseptica* was reported to have modulatory effect on dendritic cell (DC) maturation (Skinner et al., 2004)

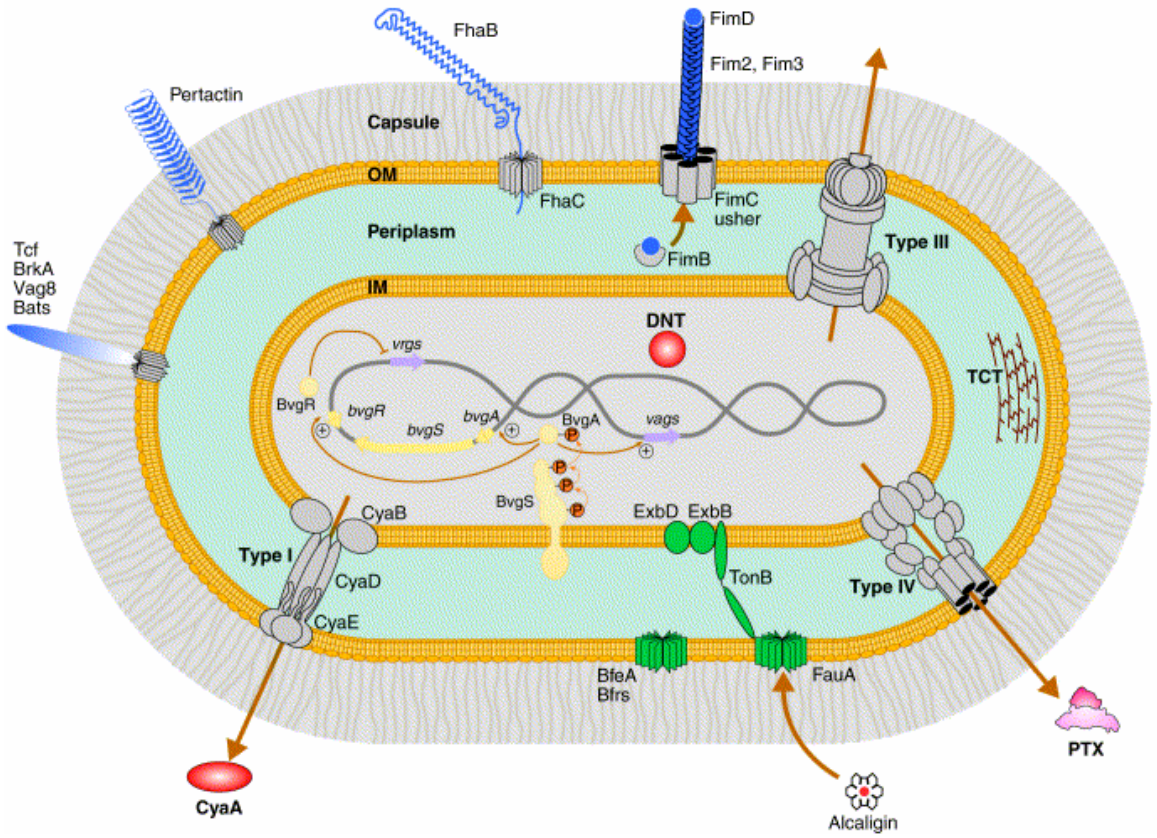

Fig. 1. Virulence factors of *B. pertussis*. Gram-negative bacteria *B. pertussis* is depicted with inner and outer membranes (IM and OM), periplasm and a capsule. The adhesins Fim, FhaB, pertactin, Tcf, BrkA, Vag8 and Bats are shown in blue; the toxins PTX, CyaA and DNT are in red; the accessory proteins FhaC, FimB, FimC, Type III, Type IV and Type I are in grey; the iron uptake systems ExbB/ExbD, TonB, FauA, BfeA and Bfrs are in green; and the regulatory systems BvgA, BvgS and BvgR are in beige. The large brown arrows represent the orientation of export and import of virulence factors and siderophores, respectively. The thinner brown arrows show the phosphorelay and the regulation circuit (Locht et al., 2001).

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

pertussis and have considerably improved safety. However, despite intensive vaccination whooping cough still presents a major health problem in some part of the world.

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

3.2 Adenylate cyclase toxin

Adenylate cyclase toxin (CyaA, ACT, AC toxin) produced by *B. pertussis* is a member of the RTX (Repeat-In-Toxin) family of bacterial pore-forming toxins. CyaA is an important pathogenic factor as it was shown that strain lacking CyaA was unable to colonize lungs and establish infection in mouse model (Goodwin & Weiss, 1990). It was shown that a large amount of CyaA stays attached to the bacterial cell surface but this toxin is not active. Instead only newly synthesized and secreted CyaA is the active form of the toxin (Gray *et al.*, 2004).

CyaA is a 1706 amino acids long protein which has two functional domains. The C-terminal domain (~1300 amino acids) possesses membrane-targeting and pore-forming activity, whereas the N-terminal domain (~400 amino acids) carries an adenylate cyclase (AC) activity. The AC domain is translocated into the cytosol of the target cell. After binding of eukaryotic calmodulin, it catalyzes unregulated conversion of cellular ATP to cAMP, a key second messenger signalling molecule. The C-terminal domain, containing glycine and aspartate-rich repeats harbouring a conserved sequence motif X-(L/I/F)-X-G-G-X-G-(D/N)-D, has an intrinsic haemolytic activity that results from its ability to form cation-selective channels in the cell membrane (Ladant & Ullmann, 1999). Recently, a third activity of this toxin was reported, which involves sustained elevation of intracellular $[Ca^{2+}]_i$, a second

messenger. This activity was demonstrated to be independent of both adenylate cyclase activity and the pore-forming activity (Fiser *et al.*, 2007).

CyaA is secreted via a type I secretion system, which formation requires accessory proteins CyaB, CyaD, and CyaE. CyaA becomes fully active after an acylation by the acyltransferase CyaC and binding of Ca^{2+} ions into RTX domain. It was shown, that acylation of Lys-983 seems to be crucial whereas acylation of Lys-860 is not necessary for CyaA activity (Masin *et al.*, 2005). Acylation is important for a tight interaction of the toxin with its receptor CD11b/CD18 integrin on target cells (El-Azami-El-Idrissi et al., 2003).

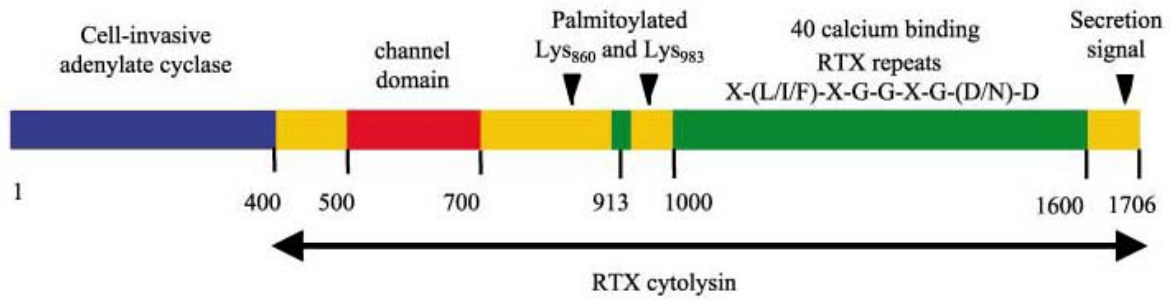

Fig. 2. Adenylate cyclase toxin (CyaA) of *B. pertussis*. (Simsova *et al.*, 2004) The N-terminal catalytic domain is ~400 residues long and possesses a cell-invasive domain with calmodulin-activated adenylate cyclase activity. The last 1300 residues represent an RTX hemolysin moiety of CyaA. The channel domain is responsible for forming of cation-selective membrane pores. Acylated Lys_{860} and Lys_{983} are part of the acylation domain, where post-transcriptional activation of the prototoxin is accomplished through CyaC-mediated covalent fatty-acylation of either of these lysins. The RTX domain carries Ca^{2+} binding repeats and mediates the interaction with β_2 integrin CD11b/CD18.

3.3 Immune system

3.3.1 Immunity in respiratory tract

The respiratory tract divides into three main segments: upper respiratory tract (including nose, pharynx), respiratory airways (larynx, trachea, bronchi and bronchioles) and lungs (respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli). The lungs itself can be segmented into conducting airways and parenchymal lung. The surface of the respiratory tract is covered by airway mucus comprises ciliated cells and secretory goblet cells. Dense networks of dendritic cells (DC) and macrophages are present in mucosa. The DC pool is composed of both the plasmacytoid DC, as well as myeloid DC subset which predominates. Specialized resident airway mucosa DC, which are important for antigen acquisition but have decreased ability of Ag presentation, are positioned directly beneath the epithelium. T cells are also abundantly found in the mucosa, namely intraepithelially (mostly expressing CD8

molecules) and within the underlying lamina propria (mostly expressing CD4 molecules). Airway epithelial cells, as key cells in the regulation of lung homeostasis, have in addition to their mucociliary clearance function also ability to secrete several families of modulatory molecules (such as NO, G-CSF, GM-CSF, ICAM) (Holt *et al.*, 2008).

3.3.2 Dendritic cells

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

3.3.2.1 Maturation of DC

The term maturation refers to a complex differentiation process whereby DC answer to an environmental stimulus and become capable of inducing adaptive immunity. Maturation is initiated in the periphery by a number of factors including pathogen-related molecules (e.g. LPS, double-stranded RNA, bacterial DNA), various pro-inflammatory and inflammatory signals (e.g. TNF-α, prostaglandins) or T cell-derived signals (e.g. CD40L)(Banchereau et al., 2000). Also innate lymphocytes like natural killer (NK) cells, natural killer T (NKT) cells or γδ T cells can trigger DC's maturation (Munz *et al.*, 2005). DC change morphologically which is manifested by a loss of adhesive structures, cytoskeleton reorganization and acquisition of a high cellular motility. Furthermore, they up-regulate the expression of co-stimulatory and adhesion molecules, secrete cytokines and chemokines, enhance antigen processing and presentation, and migrate to secondary lymphoid organs (Fig. 3).

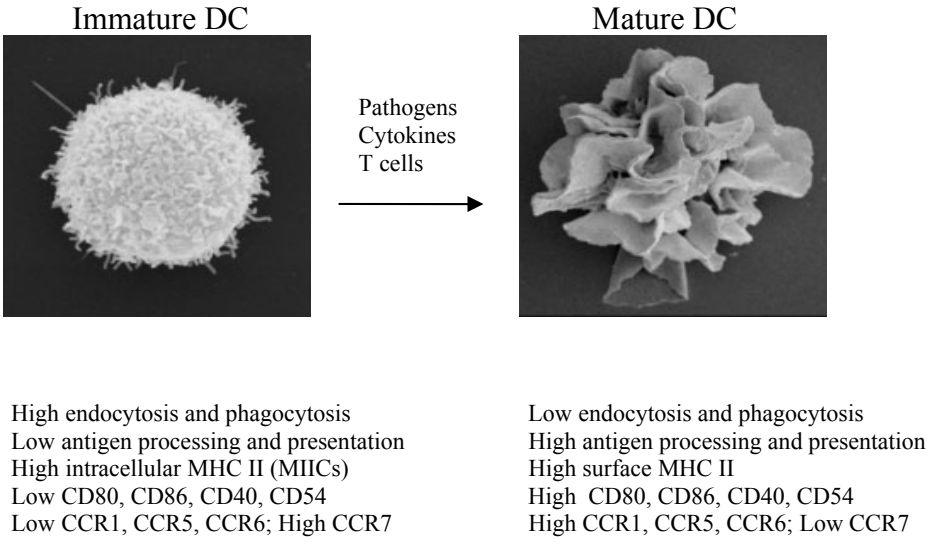

Fig. 3. Phenotypic changes during maturation of DC (Banchereau et al., 2000)(Verdijk *et al.*, 2004).

3.3.2.2 Subtypes of DC

DC arise from both myeloid and lymphoid progenitors within the bone morrow. There are two main classes of DC: conventional DC (cDC) and plasmacytoid DC (pDC). pDC generate large amounts of type I interferons and are particularly involved in response to viral infections (Colonna *et al.*, 2004). cDC differ in their phenotype, localization and function and are involved mainly in Ag presentation and activation of naïve T cells. All murine cDC express CD11c integrin on their cell surface. Moreover, additional subtypes can be distinguished according to the expression of other cell surface markers like CD4, CD8, CD11b or CD205. Spleen contains e.g. $CD4^-CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ DC. Other DC's subtypes involve $CD4^-CD8^-CD11b^+$ DC which are believed to be mature form of tissue interstitial DC or Langerhans DC found only in skin-draining lymph nodes which express high levels of langerin and E-cadherin. cDC are located virtually in all tissues and organs, where they might exert specific functions. Even though different subtypes of mouse cDC share a common capacity to present Ags to T cells, they might differ in the other functional aspects e.g. indoleamine 2,3-dioxygenase (IDO)-expressing DC (Munn, 2002) or interferon-producing killer DCs (IKDCs) (Chan *et al.*, 2006) that, however, are believed by some researchers to be a type of NK cells, rather than subtype of DC (Caminschi *et al.*, 2007, Vosshenrich *et al.*, 2007). In contrast to murine DC's subsets, relatively little is known about subsets of human DC *in vivo*. Most of the insight into human DC's subtypes was obtained from studies of their development from peripheral blood monocytes in vitro. DC can be generated also from bone-marrow by various growth factors and cytokines e.g. GM-CSF, IL-4, IL-3 (Shortman & Liu, 2002).

3.3.3 Antigen presentation

3.3.3.1 Antigen uptake by DC

DC capture Ags mainly by receptor-mediated endocytosis, macropinocytosis and phagocytosis (Watts & Amigorena, 2000) (Fig. 4).

Receptor-mediated endocytosis requires receptors that are expressed on the surface of DC, such as Fc receptors or members of the C-type lectin family, including mannose receptors. Receptor-mediated endocytosis provides a concentrating mechanism that increases the efficiency of internalization of specific ligands without taking large volume of extracellular fluid. Soluble antigens are most often engulfed after clustering of receptors in clathrin-coated pits which are formed by assembly of cytosolic coat proteins. The main assembly unit is called clathrin and forms a self-polymerising scaffold. Clathrin pits are invaginated into a cell and pinch off to form endocytic vesicles. Ligand may be released in an early endosomal compartment (e.g.transferrin) or in more acidic later endosomal compartment (e.g. low-density lipoprotein) (Conner & Schmid, 2003, Norbury, 2006).

Macropinocytosis is an actin-dependent formation of large vesicles, termed as macropinosomes, through which non-specific fluid-phase solutes are internalized. Unlike receptor-mediated endocytosis, macropinocytosis is down-regulated upon maturation in DC (Sallusto *et al.*, 1995). However, DC increase the macropinocytosis 30- to 40-min after the activation of toll-like receptors using LPS (West *et al.*, 2004). The membrane ruffling, which accompanies macropinocytosis, is normally minimal but can be stimulated with growth factors or phorbol esters (Norbury, 2006). A number of small GTPases takes part in formation of micropinosomes as well as cell-surface ruffles. For example, Rac1 and PAK1 initiate the actin polymerization, the activation of integrins is stimulated by RAP1 or RhoG, and Arf6 has effects on membrane curvature and membrane fission. (Swanson, 2008). Antigens internalized through macropinocytosis are mostly presented on MHC class II molecules. However, in case that antigens are released from internal vesicles to the cytosol of cell, they can be loaded to MHC class I molecules (Kerr & Teasdale, 2009).

Phagocytosis encompasses the engulfment of solid particles like opsonised bacteria, viruses or apoptotic and necrotic cell fragments via Fc-, complement and various scavenger receptors to form a phagosome. Although phagocytosis of bacteria is not as efficient in DC as it is probably in macrophages, it leads not only to presentation of bacterial Ags, but also drives DCs maturation (Rescigno *et al.*, 1998, Watts & Amigorena, 2000). The particles are firstly recognized, bound to the surface of cells and internalized into *de novo*-generated organelle, the phagosome. This organelle is formed undergoing a processes termed

'maturation' by fusion and limited fission events with endosomes and lysosomes to generate the mature phagolysosome. The mature phagolysosome is highly hydrophobic which serves for limiting of bacteria. The phagocytosis starts by the ligation of cell-surface receptors which bind directly to the particles or to opsonins that are detached to particle's surface. Four main classes of molecules involved in recognition are described: complement-like opsonins, scavenger receptors, a newly emerging family of epidermal growth factor (EGF)-like-repeat-containing receptors, and a highly variant receptor and opsonin, Down syndrome cell-adhesion molecule (DSCAM). The processed phagocytosed material is either loaded onto MHC class II molecules or is cross-presented on MHC class I molecules (Stuart & Ezekowitz, 2008).

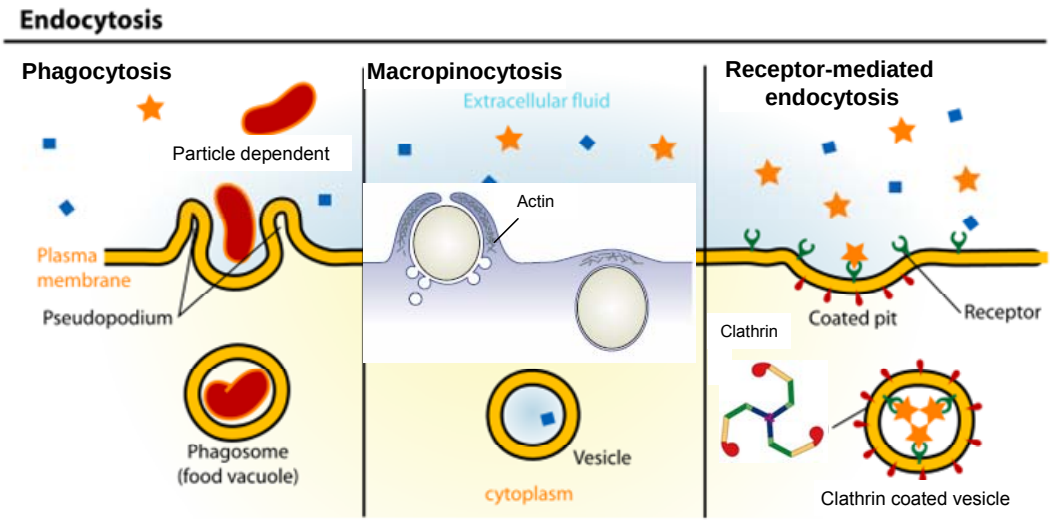

Fig. 4. Multiple portals of entry into the mammalian cell.
(http://cellbiology.med.unsw.edu.au/units/images/endocytosis_types.png; (Swanson, 2008)

3.3.3.2 Antigen processing and presentation

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

3.3.3.2.1 MHC class I-restricted antigen presentation

MHC class I molecules display on their surface short peptide fragments derived from proteins synthesized by the cell. In cytosol, proteins are ubiquitinylated and directed into proteasome.

Proteasome comprises 20S and two 19S units forming together 26S proteasome (Fig. 5.). The 19S regulator has two parts, a base and a lid. The base, which binds to 20S catalytic core, is composed of six ATPase units. These ATPases are responsible for chaperone-like activity which is used for substrates unfolding when they are channelled into the 20S core. The lid of 19S regulator is formed by eight different ATPases and its function has not been elucidated yet (Kloetzel, 2001). 20S proteasome comprises four stacked heptameric rings, two outer rings with 7 α-type subunits and two inner rings with seven β-type subunits. Only free subunits are proteolytically active: β1, β2, and β5. Each subunit has a different preference for cleavage. Proteasome subunit β1 cleaves after acidic residues (caspase-like activity), β2 cleaves after basic residues (trypsin-like activity), and β5 cleaves after hydrophobic residues (chymotrypsin-like activity) (Groettrup *et al.*, 2010). As proteasome serves as the only protease that determines the carboxyl-termini of peptides in cells, it plays an important role in antigen processing with high affinity for MHC class I binding (Murata *et al.*, 2008). In immune cells there can be two types of proteasomes: proteasome that is constitutively expressed and immunoproteasome that is formed upon exposure to inflammatory mediators like IFN-γ. IFN-γ induces formation of new protein subunits (PA28) that are incorporated into nascent 20S proteasome (Strehl *et al.*, 2005) and a new set of catalytic subunits, β1i, β2i, and β5i, collectively referred to as the immuno-subunits that replace their constitutive counterparts β1, β2, and β5, respectively. The immunoproteasome reduces the caspase-like activity and increases its chymotrypsin-like activity, which is more likely to generate C-terminus peptides, thereby enhancing its processing capacity and MHC class I presentation (Groettrup et al., 2010). Similarly to modified immunoproteasome, there is also a so called thymoproteasome, which contains proteolytically less efficient β5t subunit producing peptides with low-affinity to MHC class I molecules. However, the thymoproteasome was shown to be mainly expressed in thymus and specifically in cortical thymus epithelial cells (Murata et al., 2008).

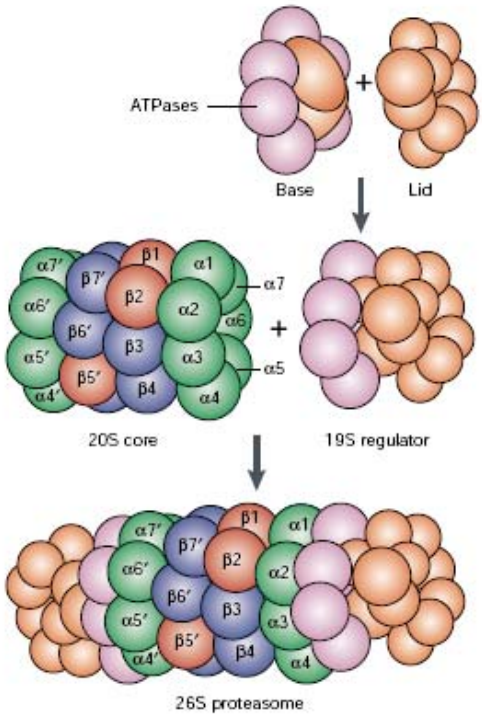

Fig. 5. Composition of the proteasome. 20S core and 19S regulator, which further consists of two multisubunit components, the ´base´ and the ´lid´, form together 26S proteasome. The three catalytic activities of proteasome, chymotrypsin-like, trypsin-like and caspase-like, are provided by 20S particle. The base of 19S regulator comprises six ATPases that have a chaperon-like activity, which mediate the correct delivery of the protein into 20S core (Kloetzel, 2001).

Peptides can be further trimmed by cytosolic peptidases on their way to endoplasmatic reticulum (ER). Newly synthesized MHC class I molecules also assemble in ER with the help of a chaperone protein, calnexin. MHC class I molecule then binds to the peptide transporter-associated protein (TAP) tapasin which forms a bridge between MHC class I molecules and TAP1/2 transmembrane transporter. Together with calreticulin and Erp57, MHC class I molecule and tapasin form MHC class I loading complex. Erp57 and calreticulin are essential to maintain the MHC class I molecule in a state receptive to peptide and also carry out a peptide-editing function. Peptides are translocated into the ER via ATP-dependent TAP1/2 and are trimmed by the ER-resident amino peptidases to 8-10 amino acids. Finally, they are loaded onto MHC class I molecules and transported from ER through Golgi apparatus to the cell surface. The simplified scheme of MHC class I-restricted Ag presentation pathway is depicted in Fig.6.

Peptides derived from endogenous proteins can be also presented on MHC class I molecules by a process called cross-presentation. DC particularly were shown to be very efficient in cross-presentation (Albert *et al.*, 1998). To date, there are no distinct entry routes known to deliver Ags preferentially for cross-presentation (Monu & Trombetta, 2007),

however some routes like macropinocytosis and phagocytosis contribute to cross-presentation more than others. In addition, FcR-mediated uptake of immune complexes, opsonized liposomes, or opsonized dead cells promote efficient cross-presentation (Guermonprez et al., 2001). It is noteworthy that autophagy, a process which cell uses to degrade cytoplasmic proteins and organelles during the maintainance of the cellular homeostasis, has been recently documented as another possible mechanism for the MHC class I-mediated cross-presentation of exogenous Ag (Crotzer & Blum, 2009).

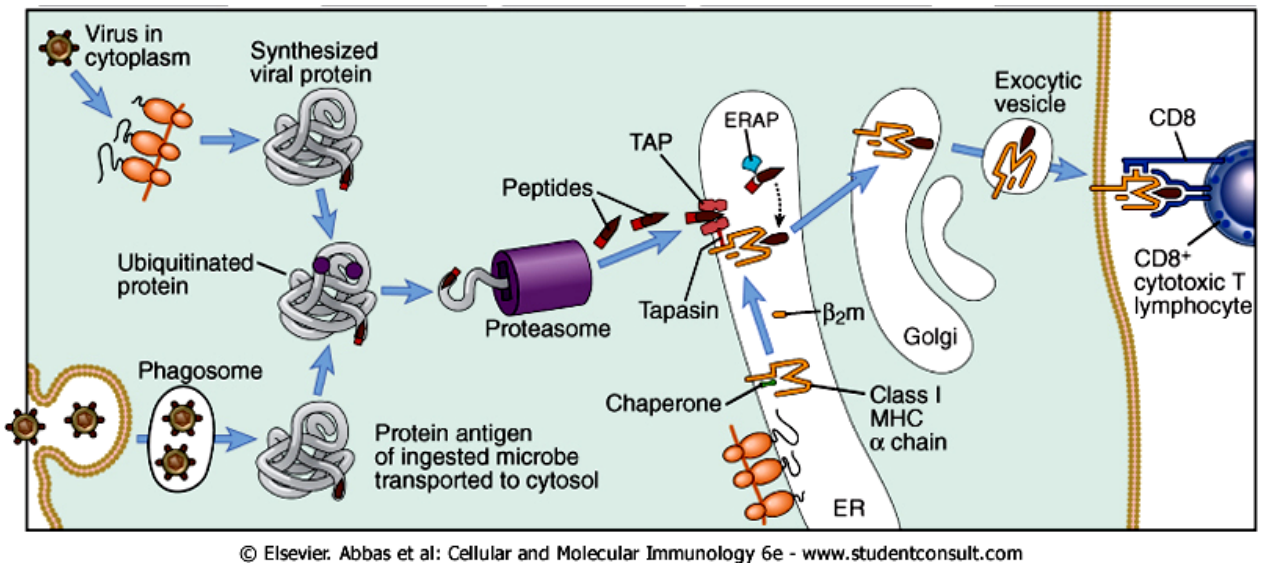

Fig. 6. MHC class I-restricted antigen presentation pathway. Ags of endogenous origin are ubiquitinilated and processed by proteasome into peptides. The peptides are consequently actively transported by TAP into endoplasmatic reticulum, where they are loaded to assembled MHC class I molecules. The complex of MHC class I molecule with bound antigen migrates through Golgi apparatus and then in exocytic vesicle to the plasma membrane where it is presented to $CD8^+$ T cells (Abbas et al., Cellular and Molecular Immunology 6th edition)

3.3.3.2.2 MHC class II-restricted antigen presentation

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

activity is also regulated by small-molecule inhibitors, such as cystatins (Honey & Rudensky, 2003).

MHC class II molecules assembly in ER with a polypeptide invariant chain (Ii) lying within the peptide-binding groove preventing so the unspecific peptide binding. Then the complex is transported through Golgi apparatus to a specific endocytic compartment MHC class II-rich compartments (MIICs) in the late endosomal pathway. In MIICs, Ii is cleaved by cathepsin S in DCs leaving a class II-associated invariant-chain peptide (CLIP) bound to MHC class II molecule. Immature DCs, specialized mainly in capturing Ags, contain high amount of preformed MHC class II molecules in MIICs and also express cystatin C which inhibits the action of cathepsin S. After maturation, the expression of cystatin C is downregulated and pH in endosomal pathway decreases thereby allowing the generation and loading of antigenic peptides (Riese *et al.*, 1996). The loading is catalyzed by a MHC class II like molecule, HLA-DM/H-2M (human/mice) which ensures the removal of CLIP and also provides an editing of weakly bound peptides. Once loaded with peptides, MHC class II molecules are transported to the plasma membrane. The binding site of MHC class II molecule has open ends, thereby longer peptides (15-35 amino acids) can be presented. The simplified scheme of MHC class II-restricted Ag presentation pathway is depicted in Fig. 7.

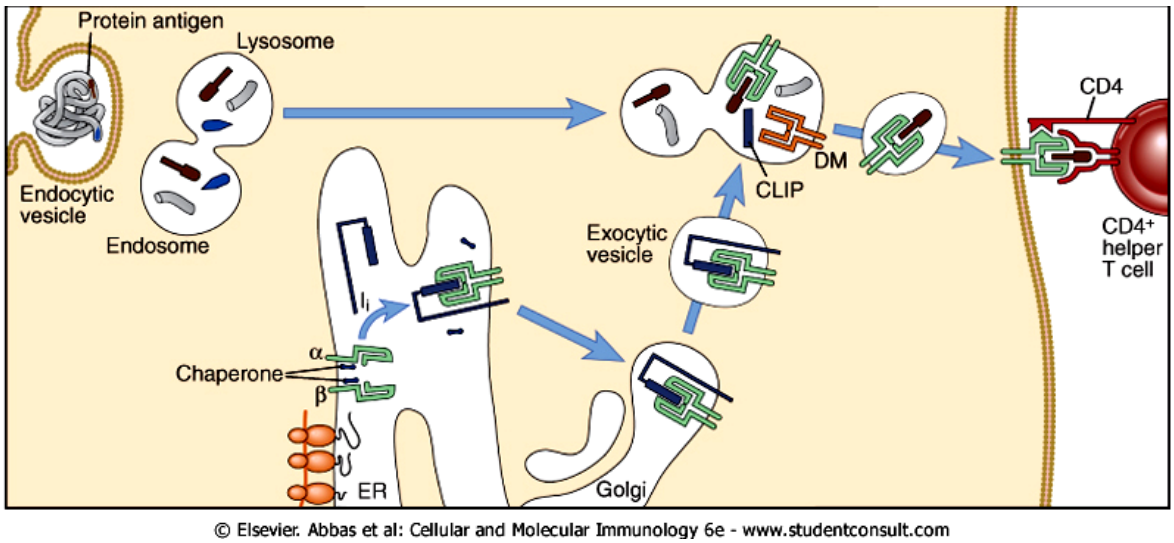

Fig. 7. MHC class II-restricted antigen presentation pathway. After synthesis in ER, three MHC class II dimers associate to form a trimer of invariant chains (Ii). The complex migrates through the Golgi apparatus to the endocytic pathway. Once reaching the acidic, protease-rich environment, Ii chain is degraded by enzymes of cathepsin family and MHC class II molecule become competent to bind Ag peptides that are mainly of exogenous origin. Ag degradation and generation of peptides in endocytic pathway require proteases, including cathepsins and endopeptidases. Once loaded with peptides, MHC class II/peptide complexes reach the plasma membrane, where they are presented to $CD4^+$ T cells (Abbas et al., Cellular and Molecular Immunology 6th edition)

3.3.3.2.3 CD1-restricted antigen presentation

In addition to MHC class I and II, a third class of MHC molecules CD1 proteins is involved in Ag presentation to T cells and also expressed by DC. CD1 proteins enable a specific recognition of an array of lipids and glycolipids that comprise the membranes of mammalian cells and microbial pathogens by T cells. There are several classes of lipid Ags that are presented by CD1 molecules, including mycobacterial mycolates, phosphatidylinositols, sphingolipids and polyisoprenoid lipids (Moody & Porcelli, 2003). According to the sequence homology, CD1 were classified into group 1 (CD1a, CD1b, CD1c, and CD1e), that is mainly expressed by DC and cortical thymocytes and group 2 (CD1d) that is expressed by various hematopoetic cells. Group 1 of CD1 molecules bind Ags of either endogenous (self-lipids, e.g., GM1) and exogenous (e.g., mycobacteria-derived lipids) origin and present them to various phenotypes of T cells (such as $CD8^+$ cytotoxic cells, $CD4^-CD8^-$ T cells, and γδ T cells (Guermonprez *et al.*, 2002).

3.3.4 T cells

T lymphocytes play a central role in controlling the adaptive immune response. They serve as crucial effector cells through antigen specific cytotoxic activity and the production of cytokines. Several different subtypes of T cells have been described based on their specific effector function: helper T cells (Th), cytotoxic T cells, memory T cells and regulatory T cells (also suppressor T cells; Treg) (Becker *et al.*, 2006, Romagnani, 2006, Sallusto *et al.*, 2004).

Helper T cells expressing co-receptor CD4 are divided into Th1, Th2 and Th17 cells. Recently, Th22 and Th9 subtypes were described (Soroosh & Doherty, 2009, Eyerich *et al.*, 2009). Th1 cells are crucial for activating macrophages to eliminate intracellular bacteria and for providing help to B cells to produce antibody. They mainly produce IFN-γ, TNF-β, IL-3, and TNF-α. Th2 cells, that are the most effective activators of B cells, produce a different set of cytokines, i.e. IL-4, IL-5, IL-9 and IL-13. Th17 cells help to recruit neutrophils to sites of infection, which is aimed mainly at extracellular pathogens. They release members of the IL-17 family such as IL-17A, IL-17E. Th9 cells were characterized as separate population from Th2 that produce in large quantities IL-9. They originated from activated $CD4^+$ T cells in the presence of TGF-β and IL-4 (Soroosh & Doherty, 2009). Similarly, Th22 were defined according to their production of cytokines, in this case mainly IL-22 and TNF-α. In contrast to Th17, Th22 produce IL-22 independently of IL-17. They are associated with epidermal repair

responses and synergize with TNF-α to induce a characteristic Th22 signature in keratinocytes (Eyerich et al., 2009).

Cytotoxic T cells (CTLs) express CD8 co-receptor on their surface. They provide protection against intracellular pathogens and are important in anti-tumour immunity. CTLs kill infected cells directly by forming pores by perforin and causing apoptosis either via secretion of granzymes or engagement of Fas receptor on the target cell. They also produce cytokines IFN-γ, TNF-α and LT-α, thereby contributing to macrophage activation.

Memory T cells, $CD4^+$ as well as $CD8^+$, function as a dynamic reservoir of Ag experienced T lymphocytes that persist long-term after an infection has resolved. They can be divided into effector memory T cells (T_{EM}) that migrate to inflamed peripheral tissues and display immediate effector function, and central memory T cells (T_{CM}) that have little or no effector function, but proliferate to effector cells in response to antigenic stimulation (Sallusto et al., 2004).

Several distinct subsets of regulatory Treg with different phenotypes and mechanisms of actions have been identified; these are Type 1 T regulatory (Tr1) cells, Th3 and $CD4^+25^+$ T cells. Type 1 T regulatory cells (Tr1) secrete high levels of IL-10 and low to moderate levels of TGF-β (McGuirk & Mills, 2002). Tr1 cells are supposed to have immunosuppressive properties, as they prevent the development of Th1-mediated autoimmune diseases or suppress immune responses to pathogens, tumors and alloantigens. Further, there are type 3 T (Th3) cells, which primarily secrete TGF-β (McGuirk & Mills, 2002). The best described regulatory T cells are represented by naturally occurring $CD4^+CD25^+$ regulatory T cells which develop in thymus and adaptive regulatory T cells which can be generated from conventional T cells in secondary lymph nodes. The regulatory effect of these Treg cells appears mechanism dependent on cell-cell contact and expression of inhibitory co-stimulatory molecule CTLA-4. They actively control the properties of other immune cells by suppressing their functional activity to prevent autoimmunity, allergy and transplant rejection. They also play a negative role in cancer (Becker et al., 2006).

3.3.5 T cell activation

Generally, three signals are necessary for efficient T cell response, these are recognition of MHC-peptide complex by T cell antigen receptors (TCRs), interaction of co-stimulatory molecules of T cell and APC, and APCs production of specific cytokines (Fig. 8A). T cell activation occurs only if TCRs recognize their specific ligands, MHC-peptide and CD28 engage co-stimulatory molecules on the surface of APC in the secondary lymphoid tissue.

After initial adhesion, the immunological synapse is formed by polarizing adhesive and signalling molecules around the site of contact between a T cell and an APC (Grakoui *et al.*, 1999) (Fig. 8B). The contact surface of the T cell is organized into two zones on the mature synapse. Central zone is known as central supramolecular activation complex (cSMAC). It contains most of the proteins important in T cell activation (TCR, CD4 or CD8 co-receptors, MHC-peptide complexes, CD28, CD2 etc.). The outer zone is called peripheral supramolecular activation complex (pSMAC). This zone is enriched of adhesive molecules LFA-1 and VLA-4, talin or transferrin receptor. Immunological synapse is considered to have an important role in regulation of T cell signalling. Whereas the activating and inhibitory molecular interactions in the immunological synapse last only seconds, the required duration of signalling, which is necessary for full T cell activation and proliferation, is in the order of hours (Bromley *et al.*, 2001, Grakoui et al., 1999).

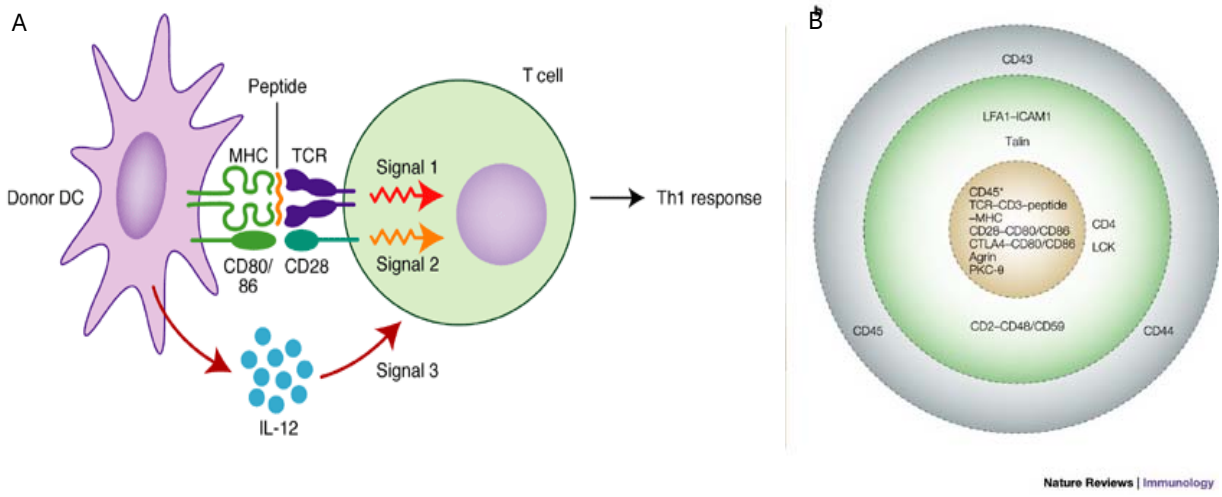

Fig. 8. T cell activation. (A) The interaction between DC and T cells involves three signals (Expert Reviews in Molecular Medicine© 2002 Cambridge University Press) **(B) Immunological synapse.** The face on view of the synapse with the characteristic 'bulls-eye' zone pattern, including the central region of the supra-molecular activation complex (cSMAC) (yellow), the peripheral ring surrounding the cSMAC (pSMAC, green) and the region distal to the synapse outside the pSMAC (dSMAC, grey) as well the molecules/ligand pairs that are found enriched within. APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein 70 (Huppa *et al.*, 2003).

3.3.6 CyaA effect on cells

3.3.6.1 Interaction of CyaA with cells of immune system

CyaA is promiscuously able to interact with a wide variety of eukaryotic cells which includes also tracheal epithelia cells inducing IL-6 due to CyaA during colonization by *B.pertussis* (Bassinet et al., 2004). CyaA was found to bind with high efficiency to target cells specifically via CD11b/CD18 (also CR3, Mac-1) (Guermonprez et al., 2001). This member of the β2-integrin family is expressed on myeloid immune cells like macrophages, neutrophils, DC or natural killer cells. These cells are predominantly targeted by CyaA *in vivo* as it was demonstrated that CyaA of *B. pertussis* inhibits phagocytic functions and induces apoptosis of macrophages (Gueirard *et al.*, 1998, Khelef *et al.*, 1993) as well as impairs chemotaxis and superoxide production in neutrophils (Friedman et al., 1987). The adenylate cyclase activity of CyaA was shown to account for these effects. However, later it was evidenced in experiments with murine macrophages J774A.1, that an increase of cAMP levels does not solely accounts for the cytotoxicity but that also a permeabilization by the pore-forming activity of CyaA contributes to the cell death (Basler *et al.*, 2006, Hewlett *et al.*, 2006). Furthermore, CyaA causes massive actin cytoskeleton rearrangements in macrophages manifested by an intensive membrane ruffling which is accompanied by an inhibition of macropinocytic uptake and complement-mediated phagocytosis (Kamanova et al., 2008).

3.3.6.2 Interaction of CyaA with DC

CyaA was shown to interact with murine bone marrow-derived DC (BMDC) as well as human peripheral blood monocytes, precursors of DC, thereby modulating the function of these cells in innate and adaptive immunity (Bagley *et al.*, 2002, Ross et al., 2004).

It was shown that CyaA inhibited LPS-induced upregulation of CD40, ICAM-1 and CD86, but not MHC class II molecules and even enhanced expression of CD80 (Boyd et al., 2005, Ross et al., 2004) in mouse BMDC treated with LPS or CpG, indicating that CyaA exhibits a differential effects on maturation of DC in the presence of a maturation stimuli. Similarly, infection of BMDC with wild type *B. bronchiseptica* significantly decreased the surface expression of CD40, but not MHC II, CD86 and CD80 when compared to CyaA-deficient bacteria (Skinner et al., 2004). No effect of CyaA on MHC II, CD86 and CD80 expression was also observed when DCs were infected with wild type or CyaA-deficient *B. pertussis* (Shumilla *et al.*, 2004, Spensieri et al., 2006). On the other side, the decrease of CD86, CD80 and CD38 expression due to treatment by CyaA was observed when DC were

infected with wild type *B. pertussis* but not with CyaA-deficient *B. pertussis* (Fedele et al., 2010).

CyaA also affects LPS-induced secretion of cytokines. It inhibits TNF-α, IL-12p70 and MIP-1α (CCL3) production in DC treated with LPS (Bagley et al., 2002, Ross et al., 2004, Skinner et al., 2004). The production of IL-12p70 was also inhibited when DC were infected with wild type *B. pertussis* (Spensieri et al., 2006, Fedele et al., 2010) as ΔcyaA mutants of *B. pertussis* released statistically significant higher amount of this cytokine which pronounced Th1 polarization. Furthermore, this group found that CyaA-mediated cAMP intoxication leads to the inhibition of IRF-1 and IRF-8 transcription factors by regulating the expression of IL-12p35 subunit which forms together with IL-12p40 mature active IL-12p70. Similarly, IRF3 expression which leads to the production of INFβ, was inhibited in DC infected by *B. pertusssis* but not in DC treated with CyaA-defective mutants (Fedele et al., 2010). CyaA also reduces the expression of IL-23 by affecting both p40 and p19 subunits gene expression (Spensieri et al., 2006) and IL-1β (Fedele et al., 2010). Ross et al. (2004) found that purified CyaA significantly augmented IL-6 and IL-10 production in LPS-treated DC (Ross et al., 2004). However, decreased levels of IL-6 production (Fedele et al., 2010) and equivalent levels of IL-10 production (Shumilla et al., 2004, Spensieri et al., 2006) were found working with ΔcyaA mutants of *B. pertussis* in infected DC compared with wild type bacteria. This may indicate that other pathogenic factors of *B. pertussis* may also induce IL-10 or IL-6 production in DC.

Mitogen activated protein kinases (MAPK) like p38 and ERK 1/2, and nuclear factor kappa B (NF-κB) pathways are involved in the regulation of DC´s maturation and cytokine production induced by TLR signalling (Rescigno et al., 1998, Sato *et al.*, 1999). Data on the regulation of NF-κB and MAPK by CyaA are limited. While Fedele et al. (2010) observed phosphorylation of p38, ERK1/2 and IkBα that induce a process allowing the activation of the NF-κB complex after a treatment of human DC with wild type *B.pertussis* but not with ΔCyaA bacterial strain, other groups have various results (Fedele et al., 2010). It was found that CyaA inhibited the p38 MAPK in BMDC infected with wild-type *B. bronchiseptica* but not by CyaA-deficient bacteria and had no effect on activation of ERK 1/2 (Skinner et al., 2004). On the other hand, Hickey et al. (2008) showed, that CyaA alone induced ERK 1/2 phosphorylation and had no effect on activation of p38 in BMDC. However, in combination with LPS, CyaA enhanced phosphorylation of p38 in these cells (Hickey *et al.*, 2008). It was also this group which found that the treatment by CyaA alone led to a significant IκBα degradation at later time-points compared with LPS controls indicating that CyaA does not

inhibit NF-κB translocation into nucleus. However, addition of CyaA did not affect IκBα degradation induced by LPS.

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

3.3.6.2 CyaA-generated cAMP signalling modulates DCs

Cyclic adenosine 3´5´-monophosphate (cAMP) is a prototypical second messenger, which modulates various physiological processes in all domains and kingdoms of life. In mammalian cells there are three known types of cAMP effector proteins: protein kinase A (PKA), guanine nucleotide-exchange protein directly activated by cAMP (EPAC) and cyclic nucleotide gated ion channels (Kamenetsky *et al.*, 2006). Recently, phosphodiesterase type 10 has been identified as a possible fourth target of cAMP signalling (Gross-Langenhoff *et al.*, 2006). In mammalian cells cAMP is degraded by phosphodiesterases.

As mentioned above, CyaA modulates the expression of costimulatory molecules as well as the production of cytokines in LPS-treated DC. To confirm that an increased level of intracellular cAMP accounts for these effects, the pharmacological cAMP-elevating agents were used. Bagley et al. (2002) showed that incubation of MDDC with a permeable cAMP analogue di-butyryl cAMP (db-cAMP) or forskolin, which activates membrane adenylate cyclases in cells, increased the expression of CD80, CD83, CD86 and HLA-DR on these cells similarly to the purified CyaA (Bagley et al., 2002). This has proved that cAMP can directly influence the expression of co-stimulatory molecules in DCs. An effect of cAMP signalling on IL-12p70 production in DC was also observed. When MDDC were infected with ΔcyaA mutant of *B. pertussis* alone, no inhibition of IL-12p70 was detected, but this could be reversed by an addition of db-cAMP to the infected cells (Spensieri et al., 2006). In other

study the modulation of DC by CyaA-generated cAMP was shown using enzymatically inactive CyaA which did not inhibit CD40 and ICAM-1 expression and TNF-α, IL-12p70 and CCL3 production CpG-stimulated BMDC (Boyd et al., 2005). All these data established very well that CyaA modulates maturation and cytokine production in DC by its ability to generate cAMP.

Direct effects of cAMP elevating agents on DC have also been studied as it is known that an increase of intracellular cAMP in immune cells generally leads to a suppression of their inflammatory function. Whereas inhibitory effects of cAMP signalling on LPS-induced TNF-α and IL-12p70 and enhancement of IL-10 production are well established in the literature (Bagley et al., 2002, Galgani *et al.*, 2004, Kambayashi *et al.*, 2001), differential effects on DC's maturation have been observed in dependence of a cAMP-elevating agent. In BMDC cAMP analogue 8-Br-cAMP, lipid mediator prostaglandin E_2 (PGE_2) or inhibitor of phosphodiesterases IBMX inhibited LPS-induced up-regulation of MHC class I, MHC class II and CD40 expression and even slightly increased the expression of CD80 and CD86 (Kambayashi et al., 2001). In contrast, the addition of 8-Br-cAMP to MDDC did not affect CD86, CD83 and HLA-DR up-regulation but inhibited the increase of CD54 expression induced by LPS (Galgani et al., 2004) suggesting that not only chemical compound but also the type and origin of DC might determine the final outcome of cAMP signalling on DC´s maturation. This might be also relevant to cAMP signalling generated by CyaA toxin.

3.3.6.3 CyaA and cAMP effects on antigen processing and presentation by DC and their capacity to stimulate T cell responses

While CyaA effects on maturation and cytokine production of LPS-stimulated DC have been well documented (Boyd et al., 2005, Ross et al., 2004, Bagley et al., 2002, Skinner et al., 2004), surprisingly, there is not much known about the effects of CyaA on Ag presentation and T cell stimulatory capacity of DC. Boschwitz at al. (1997) observed, that human monocytes were not able to stimulate $CD4^+$ T cell proliferation to tetanus toxoid when infected with wild type *B. pertussis* in contrast to cells infected with ΔcyaA mutant (Boschwitz *et al.*, 1997) suggesting that CyaA impairs T cell activation. The elevation of intracellular cAMP in BMDC by cAMP-elevating agents (8-Br-cAMP, PGE2, IBMX) caused significantly lower proliferation of peptide-specific $CD4^+$ T cells compared to LPS controls (Kambayashi et al., 2001). On the other hand, BMDC infected with wild type *B. bronchiseptica* did not decrease T cell proliferation, while the ability of bone marrow macrophages to induce T cell proliferation was diminished due to CyaA, (Siciliano *et al.*, 2006). Furthermore, it was shown in *in vivo* experiments with subcutaneously immunized

mice, that CyaA promotes the induction of Th2 and T regulatory type 1 (Tr1) cells specific for the co-administered antigen, and Fedele at al. (2010) observed that when the human DC infected with wild type *B.pertussis*, but not with CyaA-defective mutants, and cocultured with purified T lymphocytes induced IFNγ- and IL-17-producing Th effector cells. In contrast, Th2 polarization measured by IL-5 release, was significantly reduced (Fedele et al., 2010).

Another aspect which can contribute to the shift in immune response is the direct effect of cAMP generated by CyaA on T cells. Even though T cells do not express CD11b/CD18 on their surface, the increase in cytosolic cAMP levels, which can influence T cell response, were observed after the treatment with CyaA (Paccani *et al.*, 2008, Rossi Paccani *et al.*, 2009). CyaA-treated peripheral blood lymphocytes (PBMCs) dramatically increased the production of Th2 cytokines, IL-4 and IL-13, compared to PBMCs treated with the enzymaticaly inactive CyaA-AC$^-$. Furthermore CyaA exhibited a modest inhibitory effect on the production of the Th1 cytokines, IFN-γ and TNF-α in dose dependent manner (Rossi Paccani et al., 2009).

In our laboratory, we observed that the capacity of murine BMDC to initiate T cell adaptive response was impaired by CyaA activity (Adkins et al., prepared for submission) Wild type CyaA toxin incubated with LPS-treated DC significantly decreased MHC class I as well as MHC class II presentation of soluble ovalbumin compared to BMDC treated with enzymatically inactive CyaA as measured by IL-2 production of CD8$^+$ T cell line B3Z or CD4$^+$ T cell line MF2.2D9, respectively. The lower capacity of DC to stimulate IL-2 production was dependent on cAMP activity of the toxin, which was demonstrated by using db-cAMP to artificially increase cAMP levels in BMDC. Furthermore, the capacity of CyaA-treated BMDC to promote proliferation of primary naïve OVA-specific CD4$^+$ (OT-II) and CD8$^+$ (OT-I) T cells was also significantly decreased in *in vivo* adoptive transfer experiments.

Moreover, recent findings in our laboratory showed that CyaA inhibits macropinocytosis in J774A.1 macrophages (Kamanova et al., 2008). However, nothing is so far known about the effects of CyaA on Ag uptake by macropinocytosis or other endocytic pathways such as receptor-mediated endocytosis as well as on Ag processing in DC. There are studies showing that cAMP signalling might affect these processes in immune cells. Cholera toxin (CT) of *V. cholerae* and heat labile enterotoxin (LT) of enteropathogenic *E. coli* were shown to modulate MHC class II Ag processing in macrophages (Matousek *et al.*, 1998, Matousek *et al.*, 1996). CT and LT are enterotoxins that compose of A and B subunits. The enzymatically active A subunit of these toxins mediates ADP-ribosylation of the Gsα subunit of G proteins which results in the activation of adenylate cyclases and the accumulation of intracellular cAMP. It was shown that CT, and to a lesser extent LT, exhibited a negative

effect on the intracellular processing of hen egg lysosome (HEL) protein in murine macrophages (Matousek et al., 1998). CT or LT treatment of macrophages enhanced presentation of soluble HEL_{48-61} peptide to 3A9 $CD4^+$ T cell hybridoma. Furthermore, CT and LT had no effect on Ag uptake (Matousek et al., 1996). Similarly, experiments with CT-treated murine B lymphoma cells showed remarkable inhibition of their ability to present OVA, but not $OVA_{323-339}$ peptide to $CD4^+$ T-cells (Tanaka *et al.*, 1999). The authors found that the increase of intracellular cAMP level elevated pH in acidic intracellular compartments which led to reduced degradation of the Ag. Another study also showed that increased cAMP levels by cAMP-elevating agent, 8-Br-cAMP, reduced phagosome acidification in macrophages (Kalamidas *et al.*, 2006).

To date little is known about the effects of cAMP signalling on the antigen processing for the presentation on MHC class I molecules. There is a report about the enhancement of chymotrypsin-like and trypsin-like activity of proteasome by forskolin, cAMP elevating agent, in NRK (normal rat kidney) cells (Zhang *et al.*, 2007). On the other hand, Hoang and colleagues (2004) described an inhibitory effect of 8-CPT-cAMP (8-Bromoadenosine cAMP), an analogue of cAMP and some cAMP-elevating agents such as forskolin and IBMX on proteolytic proteasome functions in MSC-1 cells, mouse Sertoli cells (Hoang *et al.*, 2004). However, here are no data on the effects of CyaA toxin on the proteasomal processing functions.

3.3.7 Bacterial interference with antigen presentation

Many bacteria subvert Ag uptake, processing and/or presentation of APCs which represents an important strategy to prevent stimulation of T cell adaptive immune responses in their host. Similarly to cholera toxin of *V. cholerae* and *E. coli* enterotoxin, toxin VacA from *Helicobacter pylori* was shown to interfere with proteolytic processing of tetanus toxin and toxoid and specifically inhibits the Ii-dependent pathway of Ag presentation in autologous antigen pulsed EBV-transformed B cells (Molinari *et al.*, 1998). Intracellular bacteria *Chlamydia trachomatis* have evolved a specific mechanism for disrupting IFN-γ signalling pathways via degradation of upstream stimulatory factor (USF-1), thereby inhibiting MHC class II expression in human cells (fibroblasts, mammary epithelium cell line, HeLa) (Zhong *et al.*, 1999). It was shown that IFN-γ-induced cell surface expression of HLA-DR molecules was markedly attenuated by viable *Mycobacterium tuberculosis* in THP1, human monocyte cell line, as well as in primary monocytes (Hmama *et al.*, 1998). *M. tuberculosis* was also found to inhibit MHC class II expression and Ag processing in macrophages which was

caused by a newly identified 19-kDa lipoprotein (Noss *et al.*, 2001). Cheminay at al. (2005) demonstrated that *Salmonella enterica* inhibited Ag presentation in DC, which was dependent on proteins encoded on *Salmonella* pathogenicity island 2 (SPI2) and secreted by the type III secretion system (Cheminay *et al.*, 2005).

4. MATERIAL AND METHODS

4.1 Material

4.1.1 Technical Equipment

Bürker Counting Chamber, Marienfeld, Germany
Centrifuge Sorvall RC26 Plus, Du Pont Instruments, USA
Centrifuge Universal 32R, Hettich Zentrifugen, Germany
CO_2 Incubator, Sanyo, USA
Digital Analytic Scales, AB104-S, Mettler, Germany
Fluorescent Cytometer LSR II, BD Biosciences, USA
Freezer (-20 ºC)
Freezer (-80 ºC), Jouan, France
Inverted Macroscope IX 71, Olympus, Japan
Laminar Air Flow Cabinet Bio Star, Telstar
Microcentrifuge Bifuge Pico, Heraeus Instruments, Germany
Microplate incubator PST-60 HL plus, Boeco, Germany
Microplate reader Safire^2, Schoeller Instruments, Germany
pH-meter MV 870, Präcitronick, GDR
Power Supply for electrophoresis, Bio Rad, Inc. USA
Power Supply for SDS-PAGE, Power Pack 1000, Bio Rad, Inc. USA
Scale HF2000G, A&D Engineering, USA
Spectrophotometer for Microtiter Plates Saphire 2, Austria
Spectrophotometer S.250, Secomam, France
Ultrasonicator 4710, Cole-Parmer Instruments Co., USA
Vortex MS 1, IKA-Works, Inc., USA
Water Bath TW12, Julabo, USA
Waterbath Memmert, Germany

4.1.2 Chemicals

2-mercaptoethanol, Merck. SRN
Acetic acid, Lach-Ner, Czech Republic
Acrylamide, Biochemical Corp., USA
agarose for DNA electrophoresis,Lonza, Switzerland

Ammonium chloride, Fluka, Germany

Ampicillin, Biotika, Slovak Republic

Antibiotics, antimycotic solution (10,000 U/ml penicilin G, 10 mg/ml streptomycin, 25 µg/ml amphotericin B) 100x concentrated

APS, Sigma-Aldrich, USA

Bacterological Agar, Oxoid, UK

Bacto-tryptone, Oxoid, UK

Boric acid, Lach-Ner, Czech Republic

Bovine serum albumin (BSA), Sigma-Aldrich, USA

Bradford reagent, Bio Rad, USA

Bromphenyl Blue, Sigma-Aldrich, USA

Calcium chloride, Lach-Ner, Czech Republic

Complete Mini protease inhibitors, Roche, Germany

Coomassie Brilliant blue G250, R250, Serva, Germany

CRPG (chlor phenol red-β-D-galactopyranoside monosodium salt, Roche, USA

DEAE-Sepharose CL-6B, Sigma-Aldrich, USA

Disodium phosphate, Lach-Ner, Czech Republic

Dithiotreitol (DTT), Stratagene, USA

DMEM, prepared in UMG in Prague, Czech Republic

Ethanol, Lach-Ner, Czech Republic

Ethidium bromid, Serva, Germany

Ethylenediaminetetraacetic acid (EDTA), Lach-Ner, Czech Republic

Fetal calf serum (FCS), Sigma-Aldrich, USA

Fluorogenic substrates, BIOMOL, Germany

Glucose, 25% (w/v), prepared in UMG in Prague, Czech Republic

Glucose, Lach-Ner, Czech Republic

Glycerol, Lach-Ner, Czech Republic

Glycin, Serva, Germany

GM-CSF, produced by mouse myeloma strain P3X63

HEPES, ((2-hydroxyethyl)-1-piperazineethanesulfonic acid), Serva, Germany

Hoechst 33258, Molecular Probes Invitrogen, USA

Hoechst 33342, Molecular Probes Invitrogen, USA

Hydrogen peroxide, Lachema, Czech Republic

Chloroform, Lach-Ner, Czech Republic

Chloroquine diphosphate, Fluka, Germany

Isopropanol, Lach-Ner, Czech Republic

Isopropyl-β-D-thiagalactopyranosid (IPTG), Sigma-Aldrich, USA

Kit for Plasmid DNA Isolation, BioRad, Czech Republic

Kit QCL-100 (Quantitative chromogenic limulus amebocyte lysate kit), Cambrex, USA

Lactacystin, Sigma-Aldrich, USA

L-glutamin, 3% (w/v), prepared in UMG in Prague, Czech Republic

Lipopolysacharide from E.coli (LPS), Sigma-Aldrich, USA

Magnesium chloride, Sigma-Aldrich, USA

Magnesium sulphate, Fluka, Germany

Mannan, Sigma-Aldrich, USA

Methanol, Lach-Ner, Czech Republic

Micro BCA™ Protein Assay Kit, Pierce, USA

Monosodium phosphate, Fluka, Germany

Natrium dihydrogen phosphate, Lach-Ner, Czech Republic

Natrium hydrogen phosphate, Lach-Ner, Czech Republic

Natrium chloride, Lach-Ner, Czech Republic

Natrium pyruvate,1.1 % (w/v) prepared in UMG in Prague, Czech Republic

Non-essential amino acids, 100x, prepared in UMG in Prague, Czech Republic

O-phenylendiamine dyhydrochloride, Sigma-Aldrich, USA

Paraformaldehyde, Sigma-Aldrich, USA

PBS 10x, prepared in IMG, Czech Republic

Phenyl-Sepharose CL-4B, Sigma-Aldrich, USA

Phosphori acid, Nach-Ner, Czech Republic

Potassium acetate, Lach-Ner, Czech Republic

Potassium dihydrogen phosphate, Lach-Ner, Czech Republic

Potassium chloride, Lach-Ner, Czech Republic

Protein Standards, protein ladder (PageRuler™), Fermentas, Canada

Sodium bicarbonate 7.5 % (w/v), prepared in UMG in Prague, Czech Republic

Sodium bicarbonate, Lach-Ner, Czech Republic

Sodium carbonate, Fluka, Germany

Sodium citrate, Lachema, Czech Republic

Sodium dodecyl sulfate (SDS), Serva, Germany

Sodium fluoride, Sigma-Aldrich, USA

Sodium hydroxide, Lach-Ner, Czech Republic

Sodium sulphate, Sigma-Aldrich, USA

Sulphuric acid, Lach-Ner, Czech Republic

TEMED,(N,N,N', N'-tetramethylendiamine), Serva, Germany

Tetracyclin, Sigma-Aldrich, USA

Thiamin, Merck, Germany

TMRE (tetramethylrhodamine, ethyl ester, perchlorate), Molecular Probes Invitrogen, USA

Tris(hydroxymethyl)aminomethan (Tris), Serva, Germany

Tris(hydroxymethyl)aminomethan-hydrochlorid (Tris-HCl), Serva, Germany

Trisodium citrate, Lach-Ner, Czech Republic

Tween 20, Sigma-Aldrich, USA

Urea, Merck, Germany

VL RPMI 1640 with glutamin, prepared in UMG in Prague, Czech Republic

Yeast extract, Oxford, UK

β-meracaptoethanol, Fluka, Germany

λ-DNA, MBI Fermantas, Lithuania

4.1.3 Antibodies

Hamster anti-mouse CD11c, Allophycocyanin (APC) conjugated, eBioscience, USA

Hamster anti-mouse CD11c, Phycoerythrin (PE) conjugated, eBioscience, USA

Rat anti-mouse IL-2, Biotin labelled, BD Pharmigen, USA

Purified rat anti-mouse IL-2, BD Pharmigen, USA

4.1.4 Antigens

DQ™ Ovalbumin, Molecular Probes Invitrogen, USA

Lucifer Yellow CH, Molecular Probes Invitrogen, USA

Ovalbumin, Alexa Fluor 647® conjugate, Molecular Probes Invitrogen, USA

Ovalbumin, Fluorescein isothiocyanate (FITC) conjugate, Molecular Probes Invitrogen, USA

Transferrin, Alexa Fluor 647® conjugate, Molecular Probes Invitrogen, USA

4.1.5 Enzymes

Tab. 1: Restriction endonuclases and their buffers

Enzyme	Recognition sequence	Producer	Temperature	Buffer	0.01 % BSA
BamHI	G/GATCC	NEB (USA)	37 ºC	NEB 2	+
BsiWI	C/GTACG	NEB (USA)	55 ºC	NEB 1,2,3	-
BsrGI	T/GTACA	NEB (USA)	37 ºC	NEB 2,3	+
BstBI	TT/CGAA	NEB (USA)	65 ºC	NEB 4	-
EcoRI	G/AATTC	Fermentas (Canada)	37 ºC	NEB 1,2,3,4	-
XhoI	C/TCGAG	NEB (USA)	37 ºC	NEB 2,3,4	+

Calf intestinal alkaline phosphatase (CIP)

Ribonuclease A from bovine pancreas (Rnase), Serva, Germany

Streptavidin-HRP, RD Systems, USA

T4 DNA ligase, NEB, USA

4.1.6 Media

4.1.6.1 Media for bacterial cultures

LB medium

Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	10 g
De-ionized water	refilled to 1,000 ml

pH was adjusted to 7.0 using NaOH. The medium was sterilized in autoclave (0.12 MPa, 20 min).

MDO medium

NaH_2PO_4	3 g
Na_2HPO_4	1 g
NH_4Cl	2 g
Na_2SO_4	0.5 g
Thiamin	10 mg
Yeast extract	20 mg
Glycerol	20 mg

De-ionized water	refilled to 1000 ml

pH was adjusted to 7.0 using NaOH. The medium was sterilized in autoclave (0.12 MPa, 20 min)

SOB medium

Bacto-tryptone	20 g
Yeast extract	5 g
NaCl	0.6 g
KCl	0.2 g
De-ionized water	refilled to 1000 ml

The medium was sterilized in autoclave (0.12 MPa, 20 min). Then 1 mmol/l of $MgCl_2$ and 1 mmol/l of $MgSO_4$ were added.

4.1.6.2 Media for cell cultures

Medium for T cell lines (B3Z, MF2.2D9)

VL RPMI 1640	
FCS (inactivated at 56 ºC, 30 min)	10 % (v/v)
Glutamin	2 mmol/l
Streptomycin	0.1 mg/ml
Penicillin	1,000 U/ml
Amphotricin	0.25 µg/ml
β-mercaptoethanol	50 µmol/l
Non-essential amino acids	1 % (w/v)
Glucose	6.5 g/l

Medium for bone marrow-derived DC (BMDC)

VL RPMI 1640 or DMEM	
FCS (inactivated at 56 ºC, 30 min)	10 % v/v
Glutamin	2 mmol/l
Streptomycin	0.1 mg/ml
Penicillin	1,000 U/ml

Amphotricin	0.25 µg/ml
β-mercaptoethanol	50 µmol/l
Non-essential amino acids	1 % (w/v)
Glutamin	2 mmol/l
Natrium Pyruvate	1 mmol/l
Granulocyte-macrophage stimulating factor (GM-CSF)	20 ng/ml
$NaHCO_3$ in DMEM medium	7.5 %(v/v)

4.1.7 Buffers

Bicarbonate buffer (pH 9.6):

$NaHCO_3$	0.1 mol/l
Na_2CO_3	0.03 mol/l
De-ionized water	refilled to 1000 ml

Buffer for development of IL-2 ELISA:

Trisodium citrate	0.1 mol/l
OPD	0.46 µmol/l
H_2O_2	0.001 % (v/v)

pH 5.0 of trisodium citrate was adjusted using concentrated H_3PO_4.

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Phosphate buffered saline (PBS) (pH 7.4):

NaCl	138 mmol/l
KCl	3 mmol/l
Na_2HPO_4	12 mmol/l
KH_2PO_4	2 mmol/l

PBS – 0.5 % Tween 20:

NaCl	138 mmol/l
KCl	3 mmol/l
Na_2HPO_4	12 mmol/l
KH_2PO_4	2 mmol/l
Tween 20	0.5 (v/w)

Sample buffer for SDS-PAGE (pH 6.8):

Tris-HCl	50 mmol/l
DTT	100 mmol/l
SDS	2 % (w/v)
Bromphenyl blue	0.1 % (w/v)
Glycerol	10 % (v/v)

The solution was stored at -10 ºC. β-mercaptoethanol was added to final concentration 10 % (v/v).

Transformation buffer:

HEPES	10 mmol/l
$CaCl_2$	15 mmol/l
KCl	250 mmol/l

pH was adjusted to 6.7 using 1 M KOH and then $MnCl_2$ was added to achieve final concentration of 55 mmol/l. The solution was sterilized by filtration (Filter with a pore size of 0.22 μm)

TBE buffer (pH 8.3):

Tris-HCl	90 mmol/l
Boric acid	90 mmol/l
EDTA	2 mmol/l

TC buffer (pH 8.0):

Tris-HCl	50 mmol/l
$CaCl_2$	0.2 mmol/l

TCU buffer (pH 8.0):

Tris-HCl	50 mmol/l
$CaCl_2$	0.2 mmol/l
Urea	8 mol/l

TE buffer:

Tris	10 mmol/l
EDTA	1 mmol/l

TN-A buffer (pH 8.0):

Tris-HCl	100 mmol/l
NaCl	50 mmol/l

TN-B buffer(Equilibration buffer for Phenyl-Sepharose) (pH 8.0):

Tris-HCl	50 mmol/l
NaCl	1 mol/l

Tris-glycin buffer (pH 8.3):

Tris	25 mmol/l
Glycin	250 mmol/l
SDS	0.1 % (w/v)

UTE-A buffer (pH 8.0):

Urea	4 mol/l
Tris-HCl	50 mmol/l
EDTA	2 mmol/l

UTE-B buffer (Elution buffer for Phenyl-Sepharose) (pH 8.0):

Urea	8 mol/l
Tris-HCl	50 mmol/l
EDTA	2 mmol/l

UTN-A buffer (Equilibration buffer for DEAE-Sepharose) (pH 8.0):

Urea	8 mol/l
Tris-HCl	50 mmol/l
NaCl	120 mmol/l

UTN-B buffer (Elution buffer for DEAE-Sepharose) (pH 8.0):

Urea	8 mol/l

Tris-HCl	50 mmol/l
NaCl	200 mmol/l

Washing buffer A for Phenyl-Sepharosu (pH 8.0):

Tris-HCl	50 mmol/l
Isopropanol	60 % (v/v)

Washing buffer B for Phenyl-Sepharosu (pH 8.0):

Tris-HCl	50 mmol/l

Z – Buffer:

CRPG	4.5 mg
$MgCl_2$	1 mol/l
NP40	10 % (v/v)
β-mercaptoethanol	1 µl
PBS	refilled to 50 ml

NEB buffers (1x concentrated):

NEBuffer 1 (pH 7.0 at 25°C)

Bis Tris Propane-HCl	10 mmol/l
$MgCl_2$	10 mmol/l
DTT	1 mmol/l

NEBuffer 2 (pH 7.9 at 25°C)

Tris-HCl	10 mmol/l
$MgCl_2$	10 mmol/l
NaCl	50 mmol/l
DTT	1 mmol/l

NEBuffer 3 (pH 7.9 at 25°C)

Tris-HCl	50 mmol/l
MgCl2	10 mmol/l
NaCl	100 mmol/l
DTT	1 mmol/l

NEBuffer 4 (pH 7.9 at 25°C)

Tris-acetate	20 mmol/l
magnesium acetate	10 mmol/l
potassium acetate	50 mmol/l
DTT	1 mmol/l

T4 DNA Ligase Buffer (pH 7.5 at 25°C)

Tris-HCl	50 mmol/l
$MgCl_2$	10 mmol/l
ATP	1 mmol/l
Dithiothreitol	10 mmol/l

4.1.8 Solutions

Solutions for minipreparation and midipreparation of plasmid DNA isolation:

Solution I (S1) :

Tris-HCl (pH 8.0)	25 mmol/l
EDTA (pH 8.0)	10 mmol/l
Glucose	50 mmol/l

Solution II (S2):

NaOH	0.2 mol/l
SDS	1 % (w/v)

Solution III (S3):

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Suspension solution:

Tris-HCl (pH 7.5)	10 mmol/l
EDTA (pH 8.0)	1 mmol/l

RNAase	0.001 mg/l

Bromphenyl blue (BFB) (5 x concentrated):

Bromphenyl blue	0.25 % (w/v)
Glycerol	40 % (v/v)

Bradford reagent:

Coomassie Brilliant blue R250	0.01 % (w/v)
Ethanol	4.7 % (v/v)
H_3PO_4	8.7 % (v/v)

Cell Lysis solution:

Tris pH 8.0	20 mmol/l
NaCl	100 mmol/l
EDTA	10 mmol/l
NaF	50 mmol/l
Pyrophosphate	0.1 µmol/l

Protease inhibitor cocktail tablets (Complete Mini protease inhibitors) were added, so that one tablet was in 10 ml of lysis buffer for inhibition of protein proteolytic digestion. 100 µl of 10 % NP-40 were added just prior to using the lysis buffer

Destaining solution for SDS-PAGE:

Methanol	250 ml
Water	650 ml
Acetic acid	100 ml

Staining solution for SDS-PAGE:

Methanol	225 ml
Water	225 ml
Acetic acid	50 ml
Coomassie Brilliant blue G250	0.5 g

4.1.8 Plasmids and OVA minigene

Plasmid pT7CTACT1-233BsrGI_E5 (Fig. 9.) (Sadilkova, unpublished) which is derived from pT7CT7ACT1 (Iwaki et al., 1995) was used as a vector for insertion of ovalbumin epitopes and subsequent production of recombinant CyaA proteins by bacteria *E.coli*. The plasmid carries *cyaA* and *cyaC* genes encoding protein necessary for post-translational modification of CyaA by fatty acids. Both genes are under control of inducible *lac* promoter. The gene *cyaA* of pT7CACT1-E5 plasmid was modified by insertion of dipeptid behind 188th amino acid, which caused discontinuation of toxin ATP-bind site and consequent inactivation of CyaA enzyme activity. The plasmid carries gene for ampicillin resistance.

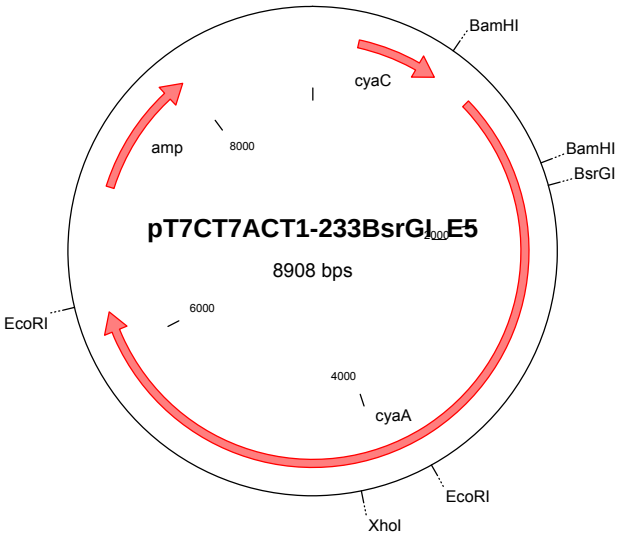

Fig. 9. Plasmid pT7CTACT1-233BsrGI_E5

Minigene (Gene_1) (Fig. 10.) cloned into plasmid 0705129pGA4 by GeneArt company. The minigene has two sequences recognized by BsiWI restriction endonuclase, which were used for cleavage of the fragment and its isolation from agarose gel. It carries $OVA_{257-264}$ (SIINFEKL) presented on murine H-2K^b MHC class I molecule that is specific for B3Z T cell hybridoma and OT-I transgenic mice. Second epitope encoded by minigene is $OVA_{258-276}$ (IINFEKLTEWTSSNVMEER) epitope presented on mouse I-A^b MHC class II molecule that is specific for TCR of MF2.2D9 T cell hybridoma. Finally there is ISQAVHAAHAEINEAGR epitope of $OVA_{323-339}$ that is specific for TCR of OT-II transgenic mice. The minigene has a sequence recognized by EcoRI, which was used during the confirmation of plasmid construction.

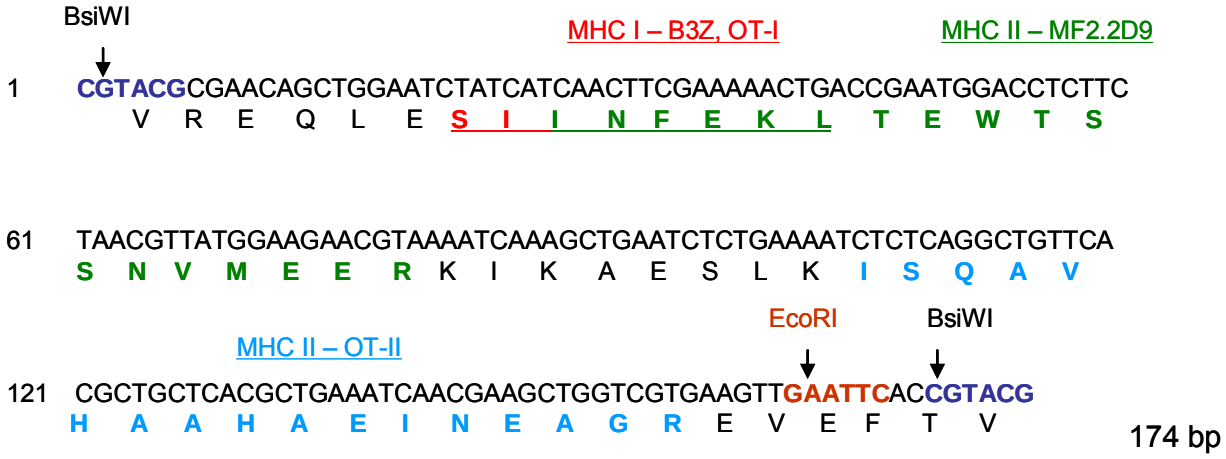

Fig.10. Minigene

Plasmid 0705129pGA4 (Fig. 11.), which was obtained from GeneArt company, carries ampicillin resistance. This plasmid contained minigene with OVA epitopes and therefore was used for amplification and after cleavage by BsiWI for consequent isolation of the minigene.

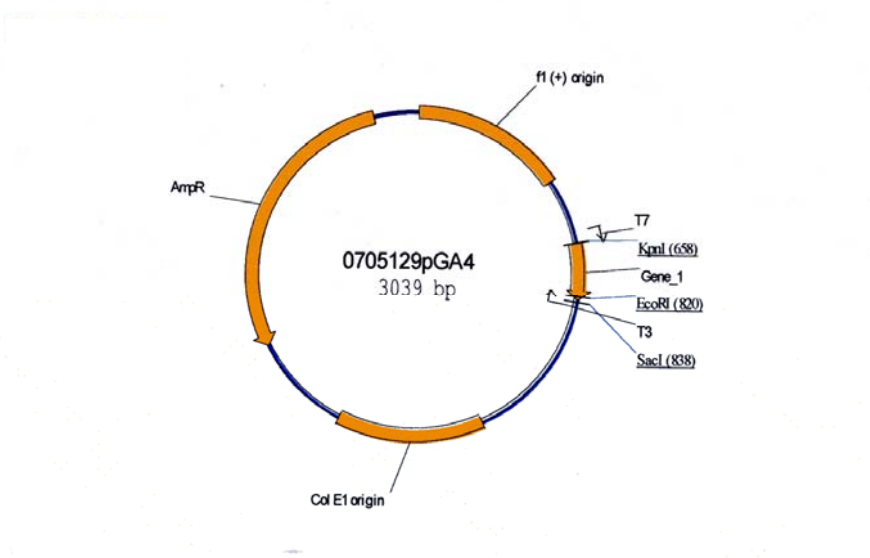

Fig. 11. Plasmid 0705129pGA4
Plasmid 0705129pGA4 with inserted fragment minigene carrying ovalbumin epitopes (the plasmid obtained from ArtGene). The plasmid carries ampicillin resistance.

Plasmid **pT7CACT1** (Osicka *et al.*, 2000) of 8.8 kb carries *cyaA* gene encoding CyaA and *cyaC* encoding protein necessary for posttranslational modification of CyaA by fatty acid. Both genes are under control of inducible lac promoter. This plasmid carries a resistance to ampicillin. The plasmid was used for production of wild-type version of CyaA.

Plasmid **pT7CACT1-E5** (Osicka et al., 2000) is derived from plasmid pT7CACT1. Dipeptide abolishing toxin ATP-binding place was inserted behind 188th amino acid of CyaA, due to this modification the CyaA enzymatic activity was abolished. This plasmid was used for production of CyaA without adenylate cyclase activity, CyaA-AC$^-$.

4.1.9 Bacterial strains

Strain *Escherichia coli* **XL1-Blue** (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´proAB lac1^q ZΔM15Tn10(Tet^r)]*) carrying resistance to tetracycline (Stratagene, USA) was used for DNA cloning.

Strain *Escherichia coli* **BL21-pMM100** is BL21 strain *E. coli B F- dcm ompT hsdS(r_B- m_B-) gal [malB$^+$]$_{K-12}$(λ^S)* transformed by plasmid pMM100 (P. Sebo) carrying gene for tetracycline resistence and gene encoding LacI^q. This strain was predominantly used for production of recombinant proteins.

4.1.10 Dendritic cells and T cell lines

Hybridoma cell lines

MF2.2D9 are T cell hybridoma non-adherent cells that express TCR for OVA$_{258-276}$ (IINFEKLTEWTSSNVMEER) epitope on mouse I-A^b MHC class II molecule (Rock *et al.*, 1993). This hybridoma cell line was kindly provided by Dr. Kenneth L. Rock (Department of Pathology, University of Massachusetts, USA).

B3Z (semi-adherent) is a lacZ-inducible CD8$^+$ T cell hybridoma specific for OVA$_{257-264}$ (SIINFEKL) presented on murine H-2K^b MHC class I molecule. B3Z represents a hybridoma generated by the vision of B3 cells (a V$_\beta$5-expressing CTL clone specific for H-2K^b/OVA) and Z.8 cells (NFAT – lacZ-inducible derivate of BW5147) (Karttunen *et al.*, 1992). The cells express β-galactosidase when its T-cell receptor engages an OVA$_{257-264}$. The hybridoma is a kind gift from Darren E. Higgins, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, USA.

Bone marrow-derived dendritic cells (DC)

Mice strains C57BL/6 were used for isolation of primary bone marrow-derived dendritic cells (DC). The cells were isolated according to (Lutz *et al.*, 1999).

Mouse myeloma strain P3X63 was used for production of GM-CSF.

4.2 Methods

4.2.1 Storage of bacterial strains

E.coli strains were kept on LB agar medium with appropriate antibiotic on a short term basis. On a long term basis, they were stored in transformation buffer with 7 % DMSO in -80 °C. Bacterial strains developed by plasmid transformation of competent cells were on a short-term basis stored on LB agar medium supplemented with appropriate antibiotic in 4 °C.

4.2.2 Preparation of *E.coli* supercompetent cells

Bacterial strains XL-1 Blue or BL21-pMM100 (4.1.9.2) were grown on agar plates supplemented with specific antibiotics for 16 – 24 hours at 37 ºC. 50 ml of SOB medium were inoculated by bacteria grown on LB agar plates, and the bacterial culture was cultivated at 37 ºC until achieving optical density (OD_{600}=0.6). The bacterial culture was consequently cooled on ice and centrifuged (6,000 g, 4 ºC, 10 min). Bacterial pellet was re-suspended in 16 ml of ice-cold transformation buffer. The cell suspension was incubated on ice (4 ºC, 10 min), repeatedly centrifuged (6,000 g, 4 ºC, 10 min), and re-suspended in 4 ml of ice-cold transformation buffer. DMSO in final concentration 7 % (v/v) was added to cell suspension, and after incubation on ice (4 ºC, 10 min) aliquots of 200 µl were frozen in liquid nitrogen. The prepared supercompetent cells were stored at temperature of -80 ºC.

4.2.3 Transformation of *E. coli* supercompetent cells by plasmid DNA

Suspension of supercompetent cells *E. coli* (50 µl) (BL21-pMM100 or XL-1 Blue) with plasmid DNA (pT7CT7ACT1-233BsrGI_E5 with or without inserted minigene, pT7CACT1, pT7CACT1-E5, or 0705129pGA4) (1 µg, approximately 0.4 µg DNA) was firstly incubated for 10 min on ice, then in warm bath (37 ºC, 5 min), and finally 3 min on ice. Pre-warmed LB medium (0.9 ml) was added to mixture, which was subsequently incubated for 1 hour at 37 ºC, so the gene for resistance to ampicillin (XL-1 Blue) or ampicillin and tetracycline (BL21-pMM100) carried by transformed plasmid DNA could be expressed. Agar LB medium supplemented with appropriate antibiotics (tetracyclin 25 µg/ml, ampicillin 100 µg/ml) was

inoculated by 50 µl of bacterial culture and the *E. coli* cells were incubated overnight (37 ºC, 16 – 20 h).

4.2.4 Isolation of plasmid DNA

4.2.4.1 Midipreparation of plasmid DNA

50 ml of LB medium supplemented with appropriate antibiotic were inoculated with bacterial colony transformed with (pT7CT7ACT1-233BsrGI_E5 with or without inserted minigene, pT7CACT1, pT7CACT1-E5, or 0705129pGA4) plasmid DNA (4.2.3). The bacterial culture was grown overnight at 37 ºC. Bacterial cells were separated by centrifugation (6,000 g, 4 ºC, 10 min) and the pellet was re-suspended in 1 ml of suspension solution. Denaturation of DNA and solubilization of cellular proteins were mediated using lysis solution (S2) (0.5 ml). The sample was mixed by a slow inversion and 5 minute incubation at room temperature followed. For DNA renaturation and SDS precipitation, 0.5 ml of neutralising solution (S3) was added. The samples were centrifuged (12,000 g, 20 ºC, 7 min) and the supernatant was removed. Consequently 3.5 ml of DNA bind buffer were added. All was mixed by an inversion. 100 – 200 µl of DNA bind particles were placed on a vacuum column. A liquid from particles was exhausted and the sample with DNA bind particles was applied on column with silica particles. The samples were washed by a wash solution (2 x 2 ml), the minicolumn was placed in the microtube and centrifuged (12,000 g, 20 °C, 1 min). The minicolumn was relocated to new microtube and centrifuged one more time to remove residual wash solution. The plasmid DNA was eluted by 50 µl of pre-warmed de-ionised water by centrifugation (12,000 g, 20 °C, 1 min). DNA bind buffer, washing buffer and silica particles are components of commercially supplied kit for plasmid DNA isolation (Top Bio, Prague, Czech Republic). Isolated plasmid DNA was stored at -20 ºC.

4.2.4.2 Minipreparation of plasmid DNA

LB medium (2 ml) supplemented with appropriate antibiotic was inoculated with one freshly grown bacterial colony (*E.coli* strain XL-1 Blue transformed with construct pT7CT7ACT1-233BsrGI_E5 carrying minigene with OVA epitopes (4.2.3)) and cultivated in shake flasks (37 ºC, 12-14 h). The bacterial culture was centrifuged (12,000 g, 20 ºC, 1 min). The cell pellet was resuspended in 100 µl of Solution I and incubated for 5 minutes at room temperature. The cells were lyzated with 200 µl of Solution II for 5 minutes on ice. Then 150 µl of Solution III were pipetted to the mixture, the content was mixed by vortex and incubated

for 15 minutes on ice. Remaining cell structures, precipitated proteins and chromosomal DNA were dispatched by centrifugation (12,000 g, 4 °C, 15 min). 400 µl of the supernatant were transferred into fresh microcentrifuge tube avoiding taking any white precipitate and the same volume of isopropanol was added to precipitate plasmid DNA. After 2 minutes on ice, the pellet of precipitated DNA was centrifuged (12,000 g, 4 °C, 15 min) and washed by 1 ml of 70 % ethanol. Microtubes were centrifuged (12,000 g, 20 °C, 3 min) and the supernatant was poured off. After 20 minutes of drying the sediment was resuspended in 40 µl of TE buffer with pancreatic RNAase (20 µg/ml) and incubated for 30 min at 70 ºC in water bath. The isolated DNA was stored at -20 ºC.

4.2.5 Manipulation with plasmid DNA

4.2.5.1 DNA digestion by restriction endonucleases

3 µl of plasmid DNA (with approximate concentration 1-2 µg) were mixed with 10x concentrated NEB buffer which was optimal for a specific restriction enzyme (Tab. 1), and if needed, the BSA was added to the mixture. Specific restriction enzymes of 1 – 2 U were added and de-ionized water was refilled to the final volume of 20 µl. The mixture was incubated for 3 hours at the temperature optimal for the restriction enzyme.

4.2.5.2 DNA ligation

DNA plasmid vector pT7CT7ACT1-233BsrGI_E5 digested by restriction BsrGI (4.2.5.1) was mixed with DNA fragments (minigene, 174 bp) (both isolated from agarose gel) in molar proportion 1:3, respectively, 3 µl of 10x concentrated and de-ionized water, which was refilled to the volume 29 µl. 1 µl of T4 DNA-ligase (5U) was added and the reaction mixture was incubated for at least 2 hours at room temperature.

4.2.5.3 DNA Electrophoresis in agarose gel

The agarose gel for electrophoresis was prepared by mixing of agarose with TBE buffer (for mass concentration 0.6 % of agarose, 1.8 g of agarose was mixed with 300 ml TBE buffer) and boiling the mixture until the agarose powder was totally melted. Ethidium bromide was added to final concentration of 0.5 µg/ml. The mixture was cooled down to approximately 50°C and poured into a prepared plastic form for preparing agarose gels. A comb was added and when the agarose gel got solid, the comb was removed and the gel with a carrier was transferred into the apparatus for electrophoresis. TBE buffer was poured to the apparatus so

that the gel was fully submerged. The sample (maximum used volume was 20 µl) with 5 µl of 5x concentrated BFB solution with a bromphenol blue was loaded to the wells of the gel. To asses the size of DNA fragments or DNA plasmids, the standard of λ DNA digested with restriction endonuclease PstI was used. Voltage of 5 V/cm^2 was used to divide fragments according to their size. The structure of agarose was used as a sieve, where large fragments move slower to positive electrode of apparatus than the smaller ones. After approximately 40 minutes, when the DNA fragments were divided, the gel was taken away and the fragments with intercalated ethidium bromide were observed under UV light.

4.2.5.4 DNA fragment isolation from agarose gel

DNA fragment (minigene with OVA epitopes or pT7CT7ACT1-233BsrGI_E5 digested by BsrGI) was excised from the agarose gel and transferred into a microtube. 900 µl of DNA binding buffer was added and the tube was placed for 10-15 minutes to 55 ºC water bath until the agarose melted. Minicolumns were attached to vacuum minifolder and 100 µl of DNA bind particles were loaded to minicolumns. Vacuum was applied until all liquid passed through the column. The dissolved gel piece containing DNA fragment was loaded onto the minicolumn. Particles with bound DNA were washed twice with 1 ml of washing buffer and the minicolumn was centrifuged (12,000 g, 20 ºC, 1 min) to remove the residual buffer. Then the minicolumn was transferred into a new mictrotube and DNA was eluted with 50 µl of pre-warmed (50 ºC) de-ionised water by centrifugation (12,000 g, 20 ºC, 1 min). The isolated DNA was stored at -20 ºC.

4.2.6 Adenylate cyclase toxin/toxoid (CyaA) isolation

4.2.6.1 Production of CyaA toxin/toxoid in bacteria

50 ml of MDO medium supplemented with antibiotic were inoculated with 2 to 3 colonies carrying suitable plasmid (4.1.8) and bacteria *E. coli* were cultivated overnight (c. 13 h) at 37ºC. Next day 500 ml of pre-warmed MDO medium supplemented with antibiotic were inoculated with 5 to 10 ml of grown bacterial culture and the cultivation of bacteria followed until the required optical density of exponential phase was obtained (OD_{600}= 0.6). The cultures were induced with IPTG (isopropyl-β-D-thiogalacopyranosid) (1 mmol/l) because of the enhanced expression of *cyaC* and *cyaA* genes in *E. coli,* which are under control of IPTG-inducible *lacZ* promoter. Bacteria were grown for another 3-4 hours, so the gene for toxin/toxoid could be expressed. The cultivation was stopped by a sudden cooling on ice.

1 ml of bacterial culture was sampled from each flask to assess the amount of produced toxin/toxoid protein. The sample was centrifuged (10,000 x g, RT, 1 min) and the cell pellet was re-suspended in 200 µl of TCU buffer. A sample (20 µl) with SDS sample buffer (5 µl, 5x concentrated) was consequently heated to 100 ºC for 5 min and loaded to 7.5 % SDS-PAGE (4.2.7).

The bacteria cultures grown in 500 ml of MDO medium, which were cooled on ice, were poured into centrifugation cuvettes and centrifuged (6000 g, 4°C, 20 min). The pellets were re-suspended in 10 ml of TC buffer and frozen at -20°C.

DNA minipreps for isolation of recombinant CyaA-OVA-AC$^-$ were tested for production of protein and for that purpose the transformed bacteria were grown in 2 ml for 4 hours. The presence of expressed toxoid was assessed as written above.

4.2.6.2 Isolation of CyaA toxin/toxoid from bacterial cultures

Growth bacterial cultures were quickly melted at 37 °C in water bath and transferred on ice. Bacterial walls were disrupted by ultrasound (3x, 50 W, 18 kHz, 4 °C, 30 seconds with 30 second brakes for cooling) and then the cells were centrifuged (6000 g, 4 °C, 3 min). The supernatant was removed into centrifuging tubes and centrifuged (13,000 g, 4 °C, 15 minutes), which caused that the toxin/toxoid present in inclusion bodies of bacteria was spinned down. The cell membranes, which were centrifuged together with inclusion bodies, were removed by washing with TUE buffer. 4.8 g of urea and 6 ml of Tris-HCl buffer pH 8 were added to the pellet (so the final concentration of urea was 8 M). The pellet was desintegrated and the mixture was incubated at room temperature for 30 minutes (occasionally the samples were mixed, so that the urea could dissolve completely). The urea extract of toxin/toxoid was gained by centrifugation (13,000 g, 4 °C, 30 minutes). NaCl, in final concentration 50 mmol/l, was added to samples, which were stored at -20 °C.

To asses the amount of toxin/toxoid in supernatant, 20 µl of samples were collected and mixed with 5 µl of 5 x concentrated sample buffer for SDS-PAGE analyse (4.2.7). This mixture was heated (100 °C, 5 minutes) and loaded to SDS-PAGE gel.

4.2.6.3 Purification of CyaA toxin/toxoid by ion-exchange chromatography on DEAE-Sepharose

The chromatographic colonies were repeatedly washed by de-ionized water and then 10 ml of DEAE-Sepharose gel were applied to each colony. The gel in colonies was washed by de-

ionized water. Colonies were equilibrated by approximately 20 ml of UTN-A till the pH of the buffer going through the colony was pH 8.0.

The urea extracts were quickly melted at 37 °C and then centrifuged (13,000 g, 4 °C, 20 min). The samples were transferred to prepared colonies where CyaA toxin/toxoid was caught. Large amount of proteins except toxin/toxoid were washed away by UTN-A buffer. Then fastened toxin/toxoid was eluted by UTN-B buffer (16 ml). The presence of protein was investigated using Bradford test and fractions containing the protein were collected. To assess the purity of protein, 20 µl of sample were mixed with 5 µl of 5x concentrated sample buffer for SDS-PAGE analyse. This mixture was heated (100 °C, 5 minutes) and loaded to SDS-PAGE gel (4.2.7).

4.2.6.4 Purification of CyaA toxin/toxoid by hydrophobic chromatography on Phenyl-Sepharose

Chromatographic colonies were washed by de-ionised water and filled with 2 ml of Phenyl Sepharose. Another washing by de-ionised water followed and a sintered glass (stored overnight in 60 % isopropanol) was inserted into each colony. Colonies were consequently equilibrated by TN-B buffer to pH 8.0. The sample containing a protein purified on DEAE-Sepharose was 4x diluted in TN-B buffer and applied on colony. The colonies with bound protein were then washed in turns by 20 ml of 60% isopropanol in 50 mM Tris-HCl pH 8 and 50 mM Tris- HCl, pH 8. Isopropanol in particular is very important for elimination of LPS in sample. This washing step was three times repeated and the elution was made by UTE-B buffer (10 ml). Bradford test was used for investigation of protein presence in samples. 20 µl of sample was collected and used for analysing purity on SDS-PAGE (4.2.7).

The whole purification using Phenyl-Sepharose was repeated one more time with the difference that the elution was made in fractions of 1 ml. Each fraction was analysed by SDS-PAGE (4.2.7). Those fractions which contained enough of the protein were mixed together.

4.2.7 SDS-electrophoresis in polyacrylamide gel (SDS-PAGE)

SDS-PAGE is a procedure for separating of molecules according to their size. The following were used for the preparation of a single gel:

Stacking 5 % gel (which was prepared by mixing 266 µl of 30 % solution of acrylamide with 1.23 ml H_2O, 200 µl 1 M Tris-HCl (pH 6.8), 60 µl 10 % (w/v) SDS, 20 µl 25 % (w/v) APS and 20 µl TEMED)

Resolving 7.5 % gel (which was prepared by mixing 1.24 ml 30 % solution of acrylamide with 1.8 ml water, 1.87 ml 1 M Tris-HCl (pH 8.8), 50 µl 10 % (w/v) SDS, 12.5 µl 25 % (w/v) APS a 12.5 µl TEMED)

The mixture of 5 % gel was poured into the prepared form and overlaid by 70 % ethanol. When the gel got firm, the ethanol was discarded and 7.5 % gel was poured over the first layer and on the top a comb was added. The gel was ready for loading of samples after another few minutes. The prepared samples were loaded into wells in gel, which were formed by the comb.

Gel in a form was submerged into a Tris-Glycin Buffer (pH 8.3) and the proteins were separated according to their size in electric field (30 V per a gel). After approximately 1 hour the buffer was discarded and the gels were put out and stained by Coomassie Brilliant Blue G250 for 1 hour (RT, gentle shaking). Then the gel was bleached by bleaching solution for SDS-PAGE (20-30 min, RT, gentle shaking). The complete gel was washed by water and dried in cellophane foil.

Unstained protein ladder (PageRuler^{TM}, Fermentas) was used for assessment of relative molecular weight of proteins.

4.2.8 Determination of CyaA toxin/toxoid concentration (Bradford-assay)

Stock of 2 mg/ml BSA was diluted in UTE-B buffer to 200 µg/ml, 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml, 0 µg/ml. The sample containing the purified protein was diluted in UTE-B buffer 20x and 30x according to visualization after purification on SDS-PAGE (4.2.7). 100 µl of diluted sample was transported into cuvette together with 900 µl Bradford reagent and incubated (15 min, RT, on dark place). Then the absorbance was measured by spectrophotometer on wave length 595 nm. The concentrations of proteins in samples were calculated using the standard curve (concentration of the BSA standards).

4.2.9 Determination of *E. coli* lipopolysaccharide (LPS) content in purified toxin/toxoid

The content of endotoxin (LPS) in purified toxin/toxoid was determined using QCL-1,000 (Quantitative chromogenic lymulus amebocyte lysate) kit. Endotoxin transforms the nonactive pro-enzyme present in this kit into an active enzyme, which is subsequently able to catalyze change of chromogenic substrate into p-nitroanilin. The amount of produced p-nitroanilin, which corresponds to the amount of present endotoxin in purified toxin, is then determined on spectrophotometer using the wave length 415 nm.

Series of dilutions of purified toxin/toxoid were prepared (1:10, 1:100, 1:1,000). Aseptic (apyrogenic) water was used for measurement of background. The series of calibration standard dilutions were prepared in the way that the scale of endotoxin concentrations was in interval 0.1 – 1 EU endotoxin/ml (1 EU of endotoxin corresponds with 83 pg of endotoxin).

The reaction ran in a microtiter plate (NUNC) on a hotplate (37 °C). 50 µl of the prepared sample (toxin/toxoid, water, standard) and 50 µl LAL (lymullus amebocyte lysate) were transferred into a single well. The mixture was consequently incubated for 10 min, and then 100 µl of substrate solution were added. After a 5 min incubation at 37 °C, the reaction was stopped by addition of 100 µl stopping solution. The amount of generated p-nitroanilin was subsequently assessed by spectrophotometer at 415 nm.

The measured absorbances of diluted standards were entered into chart in respect to endotoxin concentration (EU/ml). A calibration curve was designed

4.2.10 Generation of bone marrow-derived dendritic cells (DC)

Bone marrow-derived dendritic cells (DC) were generated according to (Lutz et al., 1999). C57BL/6 mice strain was used for the isolation of BMDC. The mice were anesthetized by chloroforms exhalations, and killed. Tibia and femur were prepared from the body using scissors and tweezers. The isolated bones were submerged into 1 % PBS, cut in joints and cleaned up from muscles by paper tissue soaked by ethanol. Ends of bones were cut and the bones were washed through by 10 ml of 1 % PBS until they got white. The cells were centrifuged (120 g, 22°C, 3 min), resuspended in 10 ml of 1 % PBS and purified by cell strainer (70 µm mesh size). The number of cells was assessed by counting in Bürker chamber. Cells were consequently re-suspended in 10 ml of DC RPMI cultivation medium and 2 x 10^6 cells were seeded per Petri dish. Cells were incubated in CO_2 incubator at 37 °C for next 3 days; on day 3 another 10 ml of DC culture medium were added. On day 6 and 8, 10 ml of DC RPMI cultivation medium with DC were taken away and the cells were centrifuged. Pellet was resuspended in fresh 10 ml of DC cultivation medium and returned to the remaining cells on Petri dishes. Experiments were done on days 7, 8 and 9. The cells were differentiated by GM-CSF (20 ng/ml) that was produced by mouse myeloma strain P3X63.

4.2.11 Handling of T hybridoma cell lines

4.2.11.1 Thawing of T cell lines

T cell lines were removed from -80 ºC and thawed quickly in 75 ºC warm water. 10 ml of pre-warmed RPMI medium (37 ºC) were applied to the tube with cells and then transferred into 50 ml Falcon tube. The cells were centrifuged (120 g, 22 ºC, 3 min), the medium was removed and the cell pellet was re-suspended in a fresh pre-warmed RPMI medium. Cells in new medium were placed into CO_2 incubator.

4.2.11.2 Passaging of T cell lines

Suspense T cell lines (MF2.2D9) were collected from tissue culture dishes, transferred to 50 ml Falcon tubes, and centrifuged (120 g, 22 ºC, 3 min). The pellet was re-suspended in 10 ml of fresh RPMI medium and according to the mass of cell pellet, part was returned to the tissue culture dish and a fresh RPMI medium was refilled to the volume 16 ml.

Adherent T cell lines (B3Z) were stripped from tissue culture dishes by cell scraper and transported into 50 ml Falcon tubes. The tubes were centrifuged (120 g, 22 ºC, 3 min) and the pellet was re-suspended in 10 ml of fresh RPMI medium. According to the mass of cell pellet, part of cells was returned to cell tissue culture dishes, and re-filled by fresh medium to 16 ml per dish.

4.2.11.3 Preparing of T cell lines for experiments

Adherent B3Z cells were stripped from tissue culture dishes by cell scraper and transported into 50 ml tubes. Suspense MF2.2D9 cells were collected to 50 ml tubes. All cells were centrifuged (120 g, 22 ºC, 3 min) and the pellet was re-suspended in RPMI. The number of cells in suspension was assessed by counting in Bürker chamber. 5 x 10^4 cells per well were used for experiments.

4.2.12 Counting of cells in Bürker chamber

A suspension of cells (c. 10 μl) was laid on Bürker chamber. The cell number of 32 squares was counted (2 x 16) and a mean figure of cells per one square was assessed. The total number of cells in one ml was calculated using the formula: n = mean number of cells per one square x 1000/0.004.

4.2.13 Toxin/toxoid dilutions

Because of the high specific activity of CyaA, the toxin needs to be stored in denaturing solutions of 8 M urea and diluted prior the addition to cell suspensions. Series of CyaA

dilutions by pre-diluting in buffer with 8 M were prepared and consequently diluted out at least 25x into the urea-free assay buffers and were added to cells rapidly. The cell samples hence receive 80 mM and lower concentrations of urea. During all experiments RPMI medium used for cultivation of DC was replaced by DMEM medium (1.9 mM Ca^{2+}) containing 200 U/ml GM-CSF to avoid uncontrollable chelation of calcium ions by the phosphate ions contained in RPMI medium, as calcium is required for CyaA activity.

4.2.14 Analyses of CyaA-OVA-AC$^-$ presentation

4.2.14.1 Verification of CyaA-OVA-AC$^-$ functionality

The experiment followed the scheme in Fig. 12. 5 x 10^4 cells per well were re-suspended in DC-DMEM medium and seeded into 96 well flat-bottomed plate. DC treated with CyaA-OVA-AC$^-$ in concentrations: 20 nM, 10 nM, 5 nM, 1 nM, 0.5 nM, 0.1 nM, 0.05 nM and 0.01 nM were co-incubated with specific T hybridoma cell line (5x10^4/well). For MHC I presentation of OVA-toxoid was used B3Z T cell line and for MHC II presentation of OVA toxoid, MF2.2D9 T cell line was added. Cells were incubated for 18 hours and sequentially the medium was discarded. Samples with MF2.2D9 T hybridoma cells were frozen in -80 °C for at least 2 h and analyzed by IL-2 ELISA. Samples with B3Z T hybridoma cells were analyzed by β-Galacosidase assay (4.2.14.2).

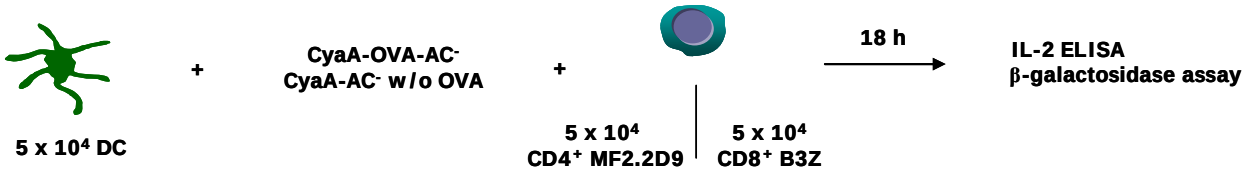

Fig. 12. Schema of experiment – Verification of CyaA-OVA-AC$^-$ functionality

4.2.14.2 β-Galacosidase assay

The medium from plates used for experiments (4.2.14.1) was discarded by flicking away and the rest of medium was drained by a paper tissue. Cells were lyzed with 100 µl of Z-buffer. The samples were incubated at 30 °C for 2 h. The reaction was stopped by adding 50 µl of 1 M glycine. The absorbance at 570 nm was read using automated microplate reader Safire 2.

4.2.14.3 IL-2 ELISA

96-well ELISA microtiter plates (Nunc-immuno, maxisorp) were coated with 1 µg/ml anti-IL-2 antibody (rat anti-mouse IL-2) diluted in bicarbonate buffer (pH 9.6) (50 µl/well) and

+incubated overnight at 4 °C. Next day the plates were washed three times with PBS-0.05 % Tween 20 by an ELISA washer and blocked with sterile PBS-1 % BSA (100 µl/well) for 2 h at 37 °C. 100 µl of sample were added to wells and plates were incubated overnight at 37 °C. The plates were washed again three times with PBS-0.05 % Tween 20 (100 µl/well). Then 0.25 µg/ml of biotinylated second antibody (rat anti-mouse IL-2, biotin labeled) diluted in PBS-1 % BSA (100 µl/well) was added and incubated for 1 h at room temperature. After the washing step, samples were further incubated with 2 µl/ml streptavidin-conjugated HRP peroxidase diluted in PBS-1 % BSA (100 µl/well) for 30 min at room temperature in a damp box. After that, plates were washed three times with PBS-0.05 % Tween 20, and ELISA was developed by adding 0.5 mg/ml of OPD substrate in citrate buffer (100 µl/well). After the 3 to 10 min of incubation the reaction was stopped by adding 100 µl of 2M H_2SO_4 per well. The absorbance was measured at 492 nm by a microplate reader Safire 2.

4.2.14.4 Presentation of CyaA-OVA-AC$^-$ by CyaA toxin-treated DC

For the experiment, whose schema is depicted on Fig.13., 1 x 10^5 of DC per well were re-suspended in DC-DMEM medium and seeded into 96 well flat-bottomed plate in triplicates. Cells in plate were then transferred into CO_2 incubator (37 °C) and left there for at least 30 min to settle down. DC were treated with various concentrations of CyaA, CyaA-AC$^-$ or cAMP elevating agent (db-cAMP, 1nM), 1 nM or 5 nM CyaA-OVA-AC for MHC I or MHC II presentation, respectively, and 0.5 mg/ml of LPS for 4 or 2 hours at 37 °C. Then the medium was discarded and 1x10^5/well of B3Z (CD8$^+$) or MF2.2D9 (CD4$^+$) T cell line were added for additional 14 hours or 16 hours, respectively. The supernatants were frozen for at least 2 hours at -80 °C and the T cell IL-2 production was determined by IL-2 ELISA.

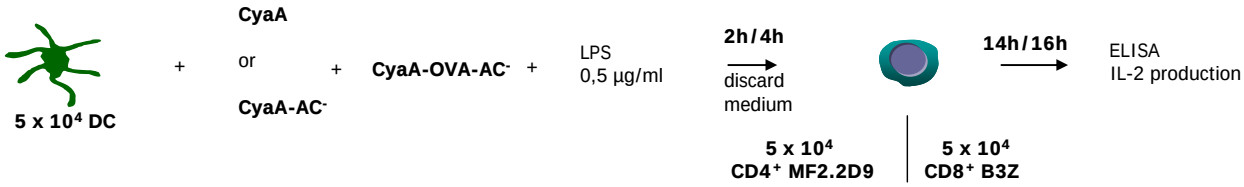

Fig. 13. Schema of experiment - CyaA effect on MHC I and MHC II presentation of CyaA-OVA-AC$^-$

4.2.14.5 Analyses of DC survival

3 x 10^5 cells per well were resuspended in DC-DMEM and seeded into non-tissue culture treated 6 well flat-bottomed plate. Cells in plate were then transferred into CO_2 incubator (37

°C, 5 % CO_2) and left there for at least 30 min to settle down. DC treated with LPS (0.5 µg/ml) were incubated with CyaA or CyaA-AC$^-$ (100 nM, 10 nM), and CyaA-OVA-AC$^-$ (2 nM) for 18 hours in 5% CO_2 at 37 ºC. Cells were sequentially stained with 40 nM TMRE (15 min, 37°C) to asses the mitochondrial potential. DC were then removed from bottoms of plate wells using cell scraper. All the volume of medium with DC was transferred into FACS tubes. 5 to 10 min before FACS analyses, the cells were stained by Hoechst 33258 (1 µg/ml). The cells in FACS tubes were analyzed with FACS and 10,000 events were collected for each sample. The simplified scheme of the experiment is depicted in Fig.14.

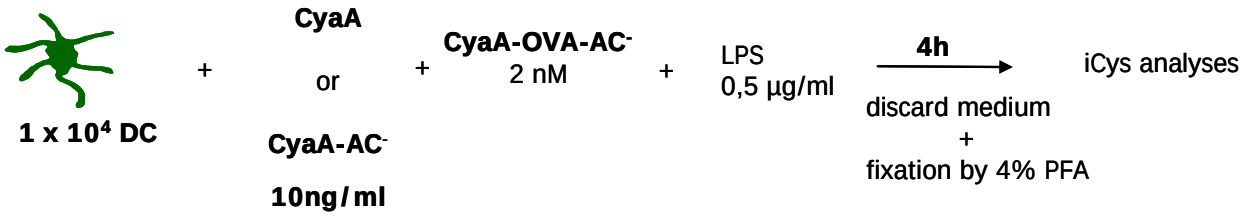

Fig. 14. Schema of experiment – Surviving of CyaA-OVA-AC$^-$-treated cells incubated with CyaA

4.2.14.6 Analyses of DC adhesion

To evaluate the loss of cell adhesion contacts from the well bottom due to effect of CyaA, DC in amount of 1 x 10^4 per well were re-suspended in DC DMEM and seeded into 96 well flat-bottomed plate. DC were incubated with wild type toxin CyaA or enzymaticaly inactive form of toxin CyaA-AC$^-$ (10 ng/ml, 100 ng/ml), CyaA-OVA-AC$^-$ (1 nM) and LPS (0.5 µg/ml) for 4 hours at 37°C in 5% CO_2. Then the medium was discarded and the cells were fixated by addition of 50 µl of pre-warmed 4 % PFA. After 15 min at 37 °C in CO_2 incubator the PFA was discarded and the cells were twice washed by 200 µl of PBS. 200 µl of PBS were added and the cells were stained by addition of 1 µg/ml of Hoechst 33342. Attached DC per a defined size of well were counted by laser scanning cytometry (iCys® Research Imaging Cytometer).

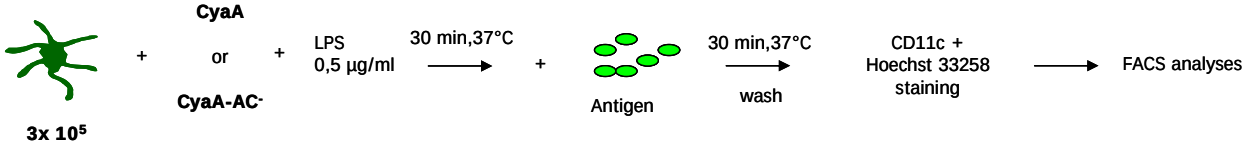

Fig. 15. Schema of experiment – Antigen uptake assay

4.2.15 Analyses of antigen uptake and processing

4.2.15.1 Ovalbumin uptake

The schema in Fig. 15. depicts the experiment where 3 x 10^5 DC/well were re-suspended in DC-DMEM medium and seeded in 96 wells plate with a conic bottom. DC were treated with 10 ng/ml or 100 ng/ml of CyaA, CyaA-AC$^-$ or mannan (1 mM) in the presence of 0.5 μg/ml LPS for 30 min at 37°C. After that, 5 μg/ml of OVA-FITC was added to cells and incubated for further 30 min at 37°C. The unspecific binding of OVA-FITC to the cells was determined by the incubation on ice. The endocytosis of OVA-FITC was stopped by adding ice-cold PBS-2 % FCS to the samples. DC were centrifuged (120 g, 22 ºC, 3 min) and consequently stained with anti-mouse CD11c-APC antibody for 25 min on ice. After an extensive washing step, cells were re-suspended in ice-cold PBS-2 % FCS and the capacity of DC to internalize OVA-FITC was assessed by flow cytometry. Hoechst 33258 (1 μg/ml) was added just before the measurement was performed to exclude the dead cells in the samples.

4.2.15.2 Lucifer yellow and trasferrin uptake

DC were re-suspended in DC-DMEM buffer, so that the number of cells in one well of 96 wells plate with a conic bottom was 3 x 10^5. DC were incubated with 10 ng/ml or 100 ng/ml of CyaA or CyaA-AC$^-$ in the presence of 0.5 μg/ml LPS for 30 min at 37 °C. After the time elapsed, fluorescently labelled transferrin 647 (10 μg/ml) or lucifer yellow (500 μg/ml) was added to cells and incubation for further 30 min (5% CO_2, 37 °C) followed. Unspecific binding of transferrin and lucifer yellow to the cell surface was assessed by incubation of cells on ice. The endocytosis of lucifer yellow or trasferrin 647 was stopped by adding ice-cold PBS-2%FCS. DC loaded with lucifer yellow or transferrin 647 were centrifuged (120 g, 22 ºC, 3 min) and consequently stained with anti-mouse CD11c-APC or CD11c-PE antibody, respectively, for 25 min on ice. After an extensive washing step, the cells were resuspended in 100 μl ice-cold PBS+2%FCS and transferred into FACS tubes. Hoechst 33258 (1 μg/ml) was added just before the measurement. The capacity of DC to internalize Ag was assessed by FACS analyses. The scheme of experiment is depicted in Fig. 15.

4.2.15.3 Antigen processing and degradation for MHC class II presentation

To asses the ability of DC treated with toxin to process and degrade Ag for MHC II presentation at a time, 1 x 10^6 DC in microtubes were treated with CyaA or CyaA-AC$^-$ in concentration 10 ng/ml or 100 ng/ml in the presence of 0.5 μg/ml LPS for 30 min at 37 °C.

Then the cells were loaded with a mixture of fluorescently labeled ovalbumins OVA-Alexa 647 (0.1 µM), and OVA labeled with BODIPY®FL dye (OVA-DQ, 0.1 µM) for additional 10 min, 20 min, 30 min, and 40 min. Unspecific binding to the cell surface was assessed by incubation of cells on ice. Every 10 minutes a sample of 1 x 10^5 cells was collected and the endocytosis was stopped by addition of ice-cold PBS-2%FCS. DC were centrifuged (120 g, 22 ºC, 3 min) and subsequently stained with anti-mouse CD11c-PE antibody for 25 min on ice. Then the cells were twice washed by PBS-2%FCS and transferred into FACS tubes. DC were prior the measurement stained with Hoechst 33258 (1 µg/ml) to exclude dead cells in sample. Fluorescence of OVA-Alexa 647 and OVA-DQ was assessed by FACS. The whole experiment is in a simplified version shown in Fig. 16A. The data were further processed using FlowJo software. Cells were firstly gated on viable cells and then on $CD11c^+$ DC. Mean fluorescence was calculated from a Alexa $647^+/DQ^+$ population without background which was assessed by treatment of OVA loaded cells on ice (Fig. 16B). A degree of processing was calculated in respect to formula in Fig. 16C.

A

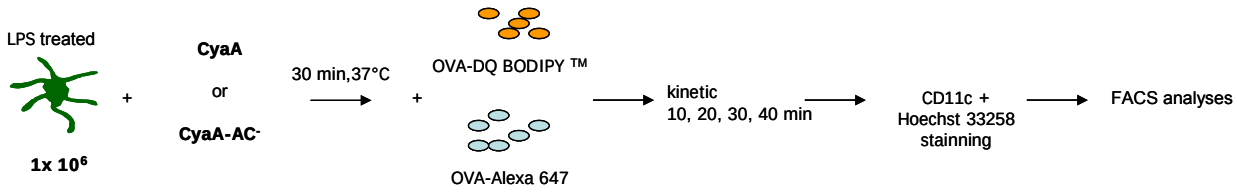

Fig. 16A: Scheme of experiment – Antigen processing and degradation for MHC II presentation

B

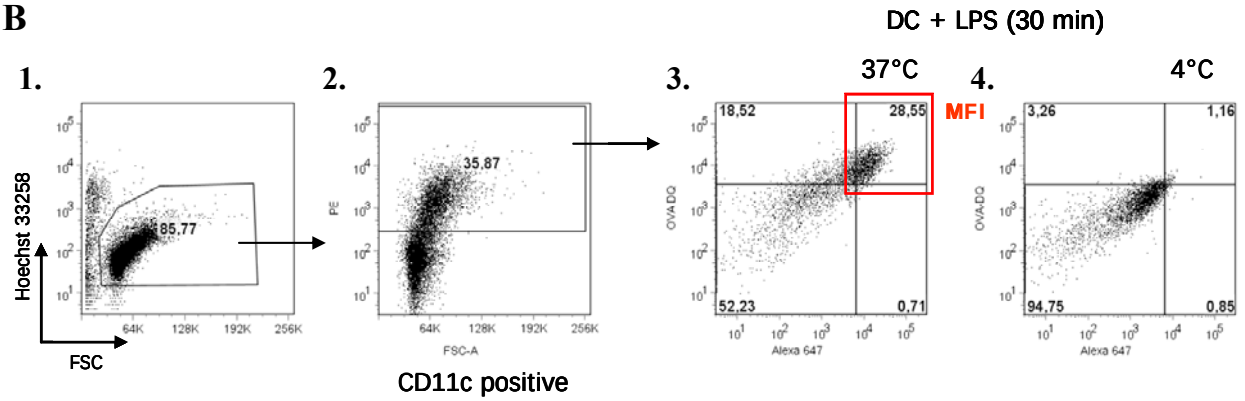

Fig. 16B Data evaluation by FlowJo software
10,000 cells were collected and analysed by fluorescence cytometery. The data were evaluated using FlowJo software. **1.** – Gating on viable population, **2.** – Gating on CD11c positive cells, **3.** – Gating on OVA-DQ^+/Alexa 647^+ population, **4.** – DC loaded with the mixture of fluorescence ovalbumins and incubated on ice were used as a negative control of unspecific binding of ovalbumins.

C

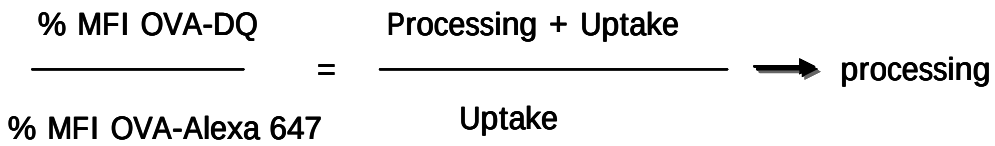

Fig. 16C Calculation of degree of OVA processing
The degree of antigen processing was assessed as a ratio of percentage of mean fluorescence of OVA-DQ to percentage of mean fluorescence of OVA-Alexa 647.

4.2.15.4 Antigen processing for MHC class I presentation

Proteolytic capacity of proteasome to degrade fluorescent substrates *in vitro* was assessed according to Zhang et al., 2007. 3×10^6 DC per sample were incubated in 6 well flat-bottomed plate overnight at 37 °C in 5% CO_2. The cells were subsequently treated with 10 ng/ml or 100 ng/ml CyaA, CyaA-AC$^-$ or 10 µM lactacystin together with 0.5 µg/ml LPS for 30 min or 1 h, respectively. DC were gently removed from wells by a cell scraper and the whole volume of each well was transferred into pre-cooled microtube. Cells were centrifuged (1,000 x g, 3 min, 4°C), washed by 1 % PBS and lyzed in lysis buffer. Protein concentration was determined by Micro BCA^{TM} Protein Assay kit (see 4.2.14.5). 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 (or Ac-Arg-Leu-Arg-AMC) fluorogenic substrates to detect caspase-like, chymotrypsin-like and trypsin-like activity of proteasome, respectively, and incubated for 1.5 h at 37 °C. The fluorescence of liberated 7-amino-4-methylcoumarin (AMC) was measured by using a microplate reader ($380_{ex}/460_{em}$). Data were corrected for the fluorescence of buffer containing fluorogenic substrates. Fig. 17 is summarizing the most important steps of the experiment.

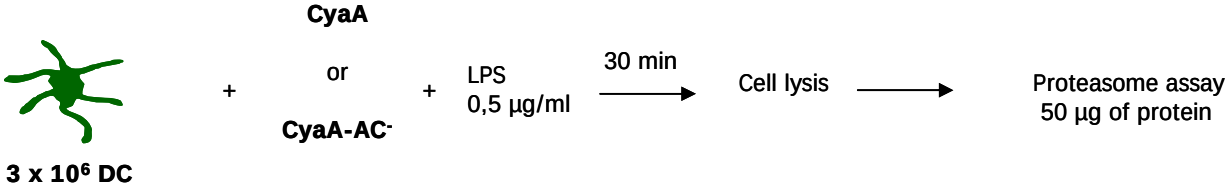

Fig. 17. Scheme of experiment – Antigen processing for MHC class I presentation

4.2.15.5 Determination of protein concentration by BCA-assay

For a determination of protein concentration was used Micro BCA^{TM} Kit, where a patented method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. A product of purple colour is formed by the chelatation of two molecules of BCA with one cuprous ion (Cu^{1+}). A strong absorbance was measured at 562 nm by spectrophotometer.

Diluted albumin (BSA) standards were prepared according to manufacture instructions. As the BCA assay is linear from concentration of 2 to 40 μg/ml, the sample was 100x diluted in PBS and 100 μl per well were transferred into 96 well flat-bottomed plate. A working reagent (WR) was prepared by mixing 25 parts of MA reagent, 24 parts of MB reagent, and 1 part of MC reagent. 100 μl of WR per well were added to the samples in wells. Everything was incubated together for 90 min at 37 °C. The absorbance was measured at 562 nm by a spectrophotometer. The concentration of proteins in samples was calculated with respect to a curve of regression equation of the BSA standards.

5. RESULTS

5.1 MHC class I and II presentation of CyaA-AC$^-$ toxoid carrying ovalbumin epitopes

5.1.1 Preparation of CyaA-OVA-AC$^-$

The aim of my work was to find out, whether CyaA toxin influences the capacity of dendritic cells (DC) to present antigens on MHC class I and II molecules and to stimulate antigen-specific T cell response. A model antigen, CyaA toxoid carrying ovalbumin epitopes (CyaA-OVA-AC$^-$) was used for the analyses of antigen presentation.

Firstly, it was necessary to construct CyaA toxoids with ovalbumin epitopes. Plasmid pT7CT7ACT1-233BsrGI_E5 (4.1.8) was used as a vector that carries gene for CyaA, which is enzymatically inactive and therefore does not elevate cAMP in cells (Sadilkova, unpublished). Sequence T/GTACA in the position 233, which is localized within the AC domain of c*yaA* gene, and which is recognized by the BsrGI restriction enzyme, was used for the insertion of minigene carrying OVA epitopes. The plasmid 0705129pGA4 obtained from GeneArt company was designed in a way that ends generated by cleavage by BsiWI were compatible with ends generated by BsrGI endonuclease, which was used for cleavage of plasmid vector (4.2.5.1). The minigene of 174 bp generated by cleavage with BsiWI (4.2.5.1) (4.2.5.4) was separated by agar electrophoresis and isolated from the gel (4.2.7). Plasmid pT7CT7ACT1-233BsrGI_E5 was linearized using BsrGI enzyme (4.2.5.1) and the alkaline phosphatase (CIP) was used (1 µl) to avoid re-ligation. Obtained fragments were ligated together (4.2.5.2). After the insertion of minigene into BsrGI cloning site in the position 233, the recognition site for the BsrGI enzyme was abolished. The plasmid with inserted minigene in the *cyaA* gene was verified by the digestion with restriction endonucleases and analysed by electrophoresis in agarose gel (4.2.6) to confirm the structure of plasmid construction. (Fig. 18., Tab. 1). Similarly, the structure of isolated vector pT7CACT1-233BsrGI_E5 without inserted OVA epitopes (CyaA-AC$^-$ w/o OVA) was verified (Fig.18., Tab. 2.).

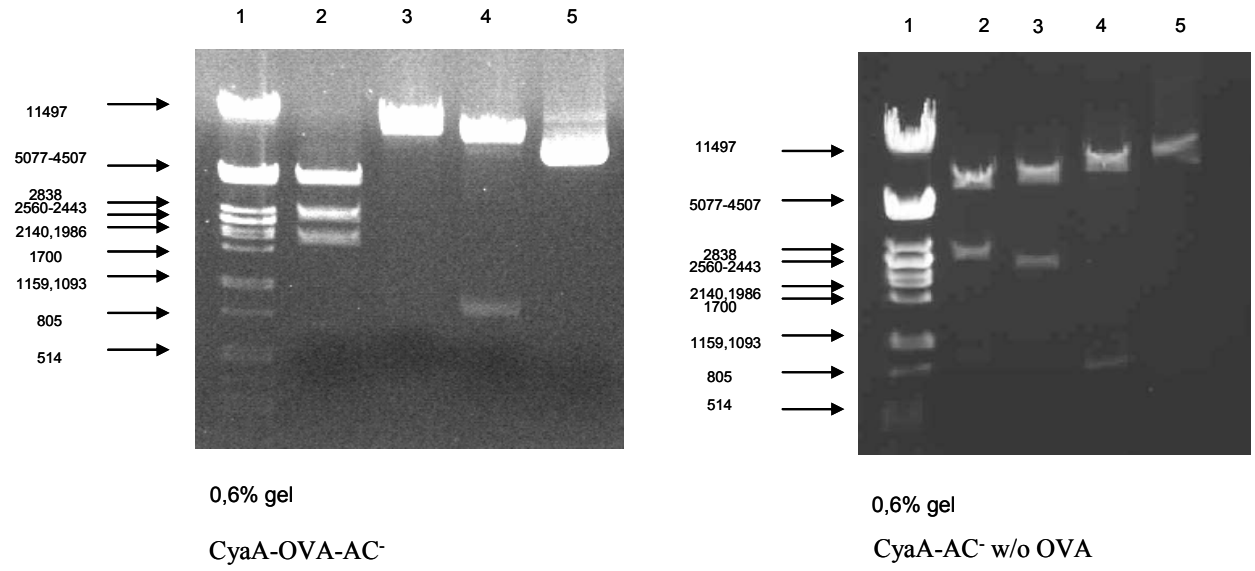

Fig. 18. Electrophoresis in 0.6 % gel of pT7CT7ACT1-233BsrGI_E5 with inserted OVA epitopes (CyaA-OVA-AC$^-$) and pT7CT7ACT1-233BsrGI_E5 (CyaA-AC$^-$ w/o OVA) 1 - Standard size λ DNA digested by PstI (used as a marker), 2 – plasmid DNA digested by EcoRI, 3 – plasmid DNA digested by BsrGI/XhoI, 4 – plasmid DNA digested by BamHI, 5 – undigested plasmid

Table 1. CyaA-OVA-AC$^-$

Plasmids used for verification	Expected sizes of fragments (bp)	Number of fragments
EcoRI	1907, 2617, 4558	3
BsrGI/XhoI	9082	1 (linearised plasmid)
BamHI	837, 8245	2

Table 2. CyaA-AC$^-$ w/o OVA

Plasmids used for verification	Expected sizes of fragments (bp)	Number of fragments
EcoRI	2617, 6291	2
BsrGI/XhoI	2329, 6579	2
BamHI	837, 8071	2

5.1.2 Expression and purification of CyaA-OVA-AC$^-$ and CyaA-AC$^-$ w/o OVA

Plasmid pT7CT7ACT1-233BsrGI_E5 encoding CyaA-OVA-AC$^-$ and the control plasmid pT7CT7ACT1-233BsrGI_E5 encoding CyaA-AC$^-$ w/o OVA were transformed into supercompetent bacteria *E.coli* BL21-pMM100 (4.2.3). Bacteria were cultivated as described

in chap. 4.2.6.1. Subsequently, bacterial cell walls were desintegrated by ultrasound and the proteins were extracted from inclusion bodies by 8 M urea (4.2.6.2). The isolated proteins were purified by ion-exchange chromatography on DEAE-Sepharose (4.2.6.3) and twice by hydrophobic chromatoghraphy on Phenyl Sepharose (4.2.6.4) to remove LPS. The size and homogeneity of CyaA derived proteins were controlled after each procedure by 7.5 % SDS-PAGE (Fig. 19.) (4.2.7). The concentration of CyaA-OVA-AC$^-$ was 750 µg/ml and CyaA-AC$^-$ w/o OVA was 500 µg/ml as determined by the Bradford method (4.2.8). Similarly, the LPS content of CyaA-OVA-AC$^-$ was 150 U/ml and CyaA-AC$^-$ w/o OVA was 100 U/ml as determined by LAL assay (4.2.9).

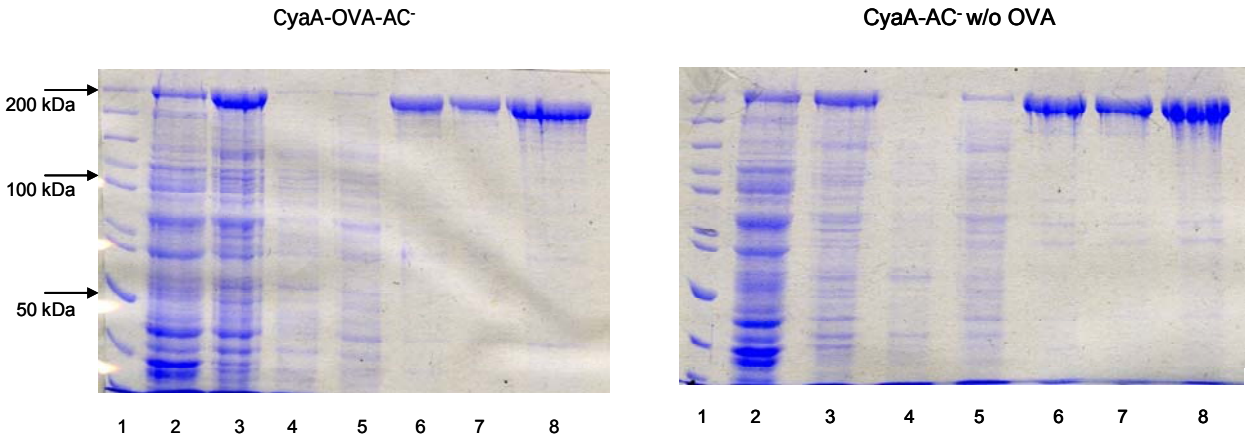

Fig. 19. Isolation and purification of CyaA-OVA-AC$^-$ and CyaA-AC$^-$ w/o OVA (SDS-PAGE Analyses) 1 – protein ladder (PageRuler^{TM}) , 2 – *E.coli* lyzates, 3 - 8M urea extracts, 4 DEAE sepharose-flow through 5, DEAE sepharose-wash step, 6 - DEAE sepharose - elution of toxoids, 7 - first phenyl sepharose purification, 8 - second phenyl sepharose purification

5.1.3 Verification of the capacity of CyaA-OVA-AC$^-$ to deliver OVA epitopes in DC for MHC class I and II presentation

After the purification of CyaA-OVA-AC$^-$ it was necessary to verify its ability to deliver OVA epitopes in bone marrow-derived DC and present them on MHC class I and II molecules to specific T cell lines B3Z and MF2.2D9, respectively. DC were incubated with CyaA-OVA-AC$^-$ or CyaA-AC$^-$ w/o OVA at the indicated concentrations (Fig. 20.) together with T cell lines for 18 hours at 37°C. The production of β-galactosidase by B3Z was measured by β-galactosidase assay (4.2.14.2) and the production of IL-2 by MF2.2D9 was measured by IL-2 ELISA (4.2.14.3.).

The results in Fig. 20 show that the herein constructed and purified CyaA-OVA-AC$^-$ toxoid (blue colour) is able to deliver OVA epitopes in DC and present them on MHC class I

molecules to B3Z T cell line, at concentration of 0.5 nM. Similarly CyaA-OVA-AC$^-$ delivered OVA epitope for presentation on MHC class II molecules to MF2.2D9 cell line, at the concentration of 1 nM. As expected, the protein CyaA-AC$^-$ w/o OVA (green colour) did not induce any β-galactosidase in the case of MHC I presentation, in the case of presentation on MHC class II molecules, IL-2 production was detected slightly above the background levels at higher concentrations, above 10 nM, of the toxoid.

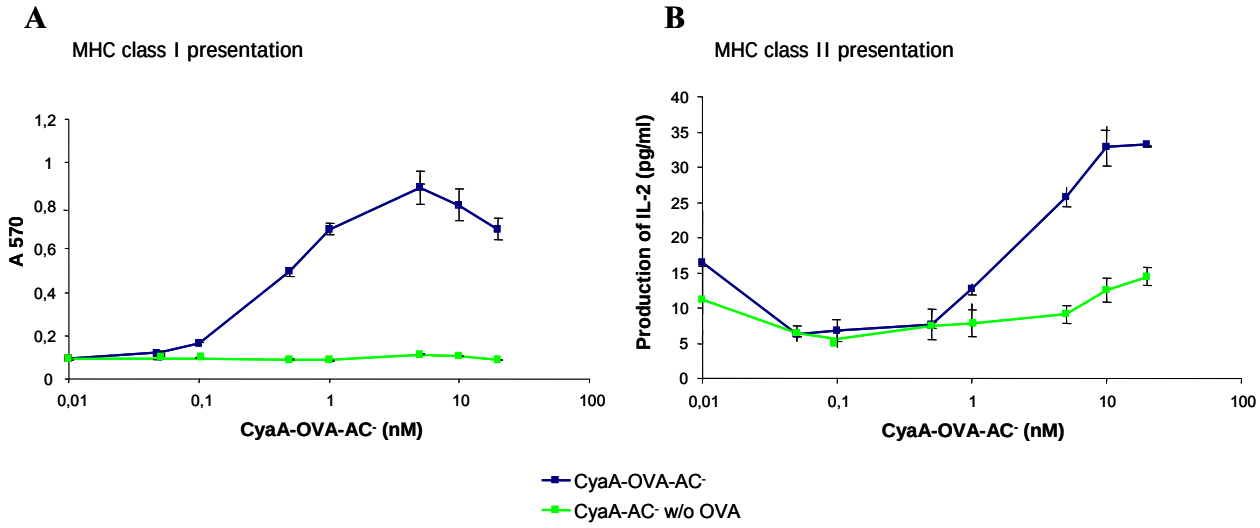

Fig. 20. Verification of MHC class I and II presentation of OVA toxoid (CyaA-OVA-AC$^-$). (A) Presentation of CyaA-OVA-AC$^-$ on MHC class I molecules by DC to B3Z T cell line. Production of β-galactosidase by B3Z was analysed by β-galactosidase assay. **(B)** Presentation of CyaA-OVA-AC$^-$ on MHC class II molecules by DC to MF2.2D9. IL-2 production was analysed by IL-2 ELISA and calculated to pg/ml of IL-2 with respect to IL-2 calibration curve. (■) CyaA-AC$^-$ w/o OVA was used as a negative control.

5.1.4 Expression and purification of wild type CyaA toxin and detoxified CyaA-AC$^-$

Plasmids pT7CACT1 and pT7CACT1-E5 (4.1.8) were used for expression of CyaA toxin and enzymatically inactive version of CyaA, CyaA-AC$^-$. Bacterial strain *E.coli* BL-21-pMM100 was transformed (4.2.3) by these plasmids and the expression of the genes was induced by addition of IPTG (4.2.6.1). The proteins were isolated from desintagrated *E. coli* cells (4.2.6.2) and purified firstly by ion-exchange chromatography on DEAE-Sepharose (4.2.6.3) and then twice by hydrophobic chromatoghraphy on Phenyl Sepharose (4.2.6.4). The analysis of isolated and purified toxins by SDS-PAGE (4.2.7) is shown in Fig. 21. The protein concentration was determined by Bradford method (500 μg/ml CyaA, 1000 μg/ml CyaA-AC$^-$)

(4.2.8). The content of LPS was measured 250 U/ml of LPS in CyaA and 150 U/ml in CyaA-AC$^-$ by LAL assay (4.2.9).

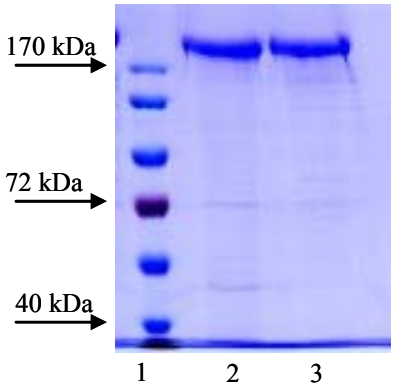

Fig. 21. Analysis of isolated and purified toxins by SDS-PAGE.
1 – protein ladder (PageRuler^{TM}) , 2 – CyaA-AC$^-$ second phenyl sepharose purification, 3 - CyaA second phenyl sepharose purification

5.1.5 CyaA effects on CyaA-OVA-AC$^-$ toxoid presentation by LPS-stimulated DC to OVA-specific T cell lines

In our laboratory, we found that soluble OVA-loaded DC treated with 10 ng/ml of CyaA toxin in the presence of LPS exhibited a reduced capacity to stimulate OVA-specific CD8$^+$ and CD4$^+$ T cells hybridoma as well as primary T cells (Adkins et al., prepared for submission). Therefore we were interested whether CyaA is also able to affect the presentation of CyaA-OVA-AC$^-$ toxoid as a different model antigen. Towards this aim LPS-treated DC were incubated with wild type CyaA or enzymatically inactive CyaA-AC$^-$ at the indicated concentrations (Fig. 22.) in the presence of OVA toxoid as a model antigen at the concentration of 1 nM (MHC class I presentation) and 2 nM (MHC class II presentation) (4.2.14.4), respectively. After 2h, in the case of MHC class II presentation, MF2.2D9 T cell hybridoma was added and the cells were incubated for further 16 hours. In the case of MHC class I presentation, B3Z T cell hybridoma was added after 4 hours and the incubation lasted for another 14 hours. Both L-2 productions in supernatants of T cell hybridomas were measured by IL-2 ELISA after overall time incubation of 18 h (4.2.14.3).

Fig. 22. A and B show that CyaA in comparison to enzymatically inactive CyaA-AC$^-$, which is not able to produce cAMP, decreases the IL-2 production by MF2.2D9, as well as by B3Z T cells hybridoma, already at a toxin concentration of 1 nM suggesting that CyaA decreases antigen presentation on MHC class I as well as MHC class II in DC. These results were confirmed by 3 independent experiments (Fig. 22. C and D), where 10 ng/ml of CyaA

inhibited IL-2 production by ~65 % in B3Z and by ~70 % in MF2.2D9. Interestingly, already 1 ng/ml CyaA decreased MHC class I presentation by 60 %.

Furthermore, CyaA-OVA-AC$^-$-loaded DC were treated with cAMP analog, db-cAMP (1 mM) to determine whether the effects of CyaA on CyaA-OVA-AC$^-$ toxoid presentation were due to increased levels of cAMP as it was observed that cAMP-elevating agents inhibited MHC class II antigen presentation by DC (Kambayashi et al., 2001). As shown in Fig. 23, db-cAMP decreased the IL-2 production by B3Z by ~60 %, while the reduction in IL-2 production by MF2.2D9 was only by ~30 % confirming the fact that cAMP induced due to CyaA accounts for the inhibitory effects of the toxin on the antigen presentation by the DC.

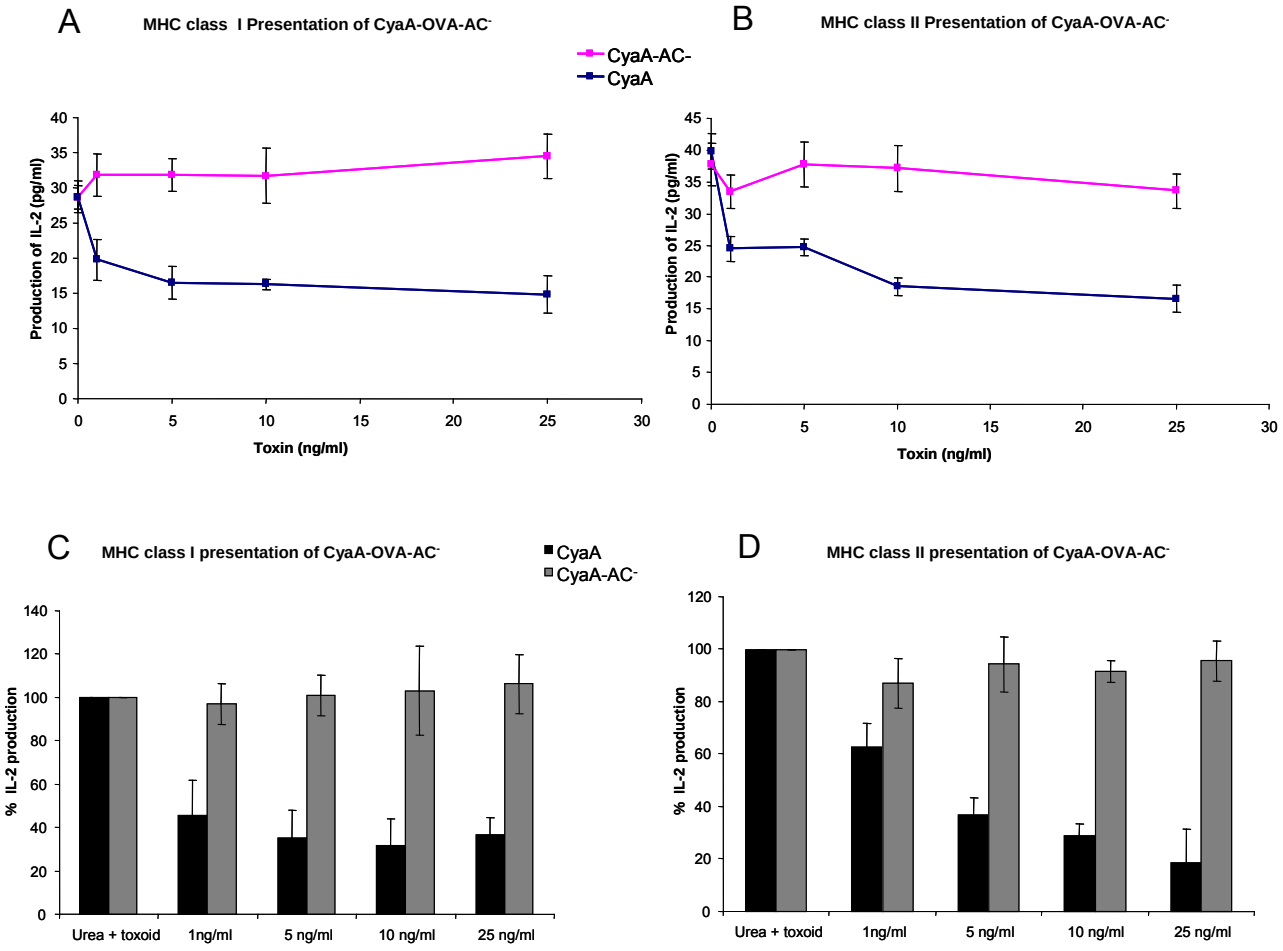

Fig. 22. Analyses of CyaA effect on MHC class I and II presentation of CyaA-OVA-AC$^-$ toxoid by DC. 1x10^5 DC/well were incubated with CyaA-OVA-AC$^-$ at the concentration of 1 nM **(A)(C)** and 5 nM **(B)(D)** together with the indicated concentrations of CyaA or CyaA-AC$^-$. After 4 hours, B3Z T cell hybridoma was added and incubated for further 16h **(A)(C)** or after 2 hours, MF2.2D9 T cell hybridoma was added and incubated for further 14h **(B)(D)**. The IL-production in the supernatant was assessed by IL-2 ELISA.
(A)(B) Data are representative of 3 independent experiments. Cells treated with CyaA are marked by (■), DC treated with CyaA-AC$^-$ by (■). **(B)(D)** Quantitative analyses of the data from 3 independent experiments as mean ± SD. The percentage of IL-2 production was calculated from the raw data in respect to cells incubated only with buffer (100%). CyaA treated DC are marked by (■), CyaA-AC$^-$ treated DC by (■).

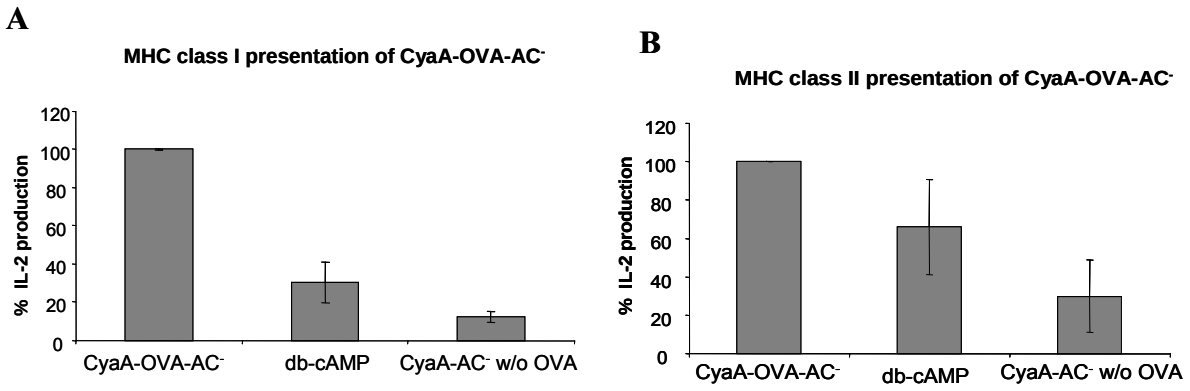

Fig. 23. MHC class I and II presentation of CyaA-OVA-AC$^-$ by DC treated with db-cAMP
1x10^5/well DC were incubated with db-cAMP (1 mM) in the presence of CyaA-OVA-AC$^-$ or CyaA-AC$^-$ w/o OVA (1 nM) for MHC class I **(A)** and 5 nM for MHC class II **(B)** presentation. The presentation of CyaA-OVA-AC$^-$ was set to 100 %. Results are means ± SD of 3 independent experiments.

5.1.6 Survival of LPS-treated DC after CyaA treatment

It was reported that CyaA causes cell death of macrophages and monocytes (Khelef et al., 1993, Masin et al., 2005, Basler et al., 2006) which could be one of the reasons why the IL-2 production by T cells was decreased. To exclude this possibility LPS-treated DC were incubated with the wild type CyaA in various concentrations or enzymatically inactive CyaA-AC$^-$ in the presence of 2 nM CyaA-OVA-AC$^-$ toxoid as a model antigen for 18 hours (4.2.14.5).

DC were stained by TMRE to visualize the decrease of mitochondrial potential in apoptotic cells and prior the measurement by flow cytometry, Hoechst 33258 was used for staining of apoptotic and necrotic cells. 10,000 cells were analyzed by fluorescent cytometry. As live cells were detected those cells, which did not decrease mitochondrial potential, visualized by TMRE and were not stained by Hoechst 33258 (Hoechst 33258$^-$/TMRE$^+$).

As demonstrated by the dotplots in Fig. 24 A, the number of DC treated with 10 ng/ml of CyaA and the model antigen CyaA-OVA-AC$^-$ toxoid was similar to the number of DC treated only with CyaA-OVA-AC$^-$. In contrast, cells treated with 100 ng/ml of CyaA were by 20 % less viable showing the potential of CyaA to cause cell death. Quantitative analyses further confirm the data. In Fig. 24 B, the percentage of live cells treated with 10 ng/ml of CyaA was comparable with the percentage of live cells treated with 100 ng/ml of detoxified, enzymatically inactive version of toxin, CyaA-AC$^-$. 100 ng/ml CyaA caused death of ~20 %

DC and therefore demonstrated the toxicity of CyaA in higher concentrations. These results show that CyaA at a concentration of 10 ng/ml does not cause the death of DC (Fig. 24B)

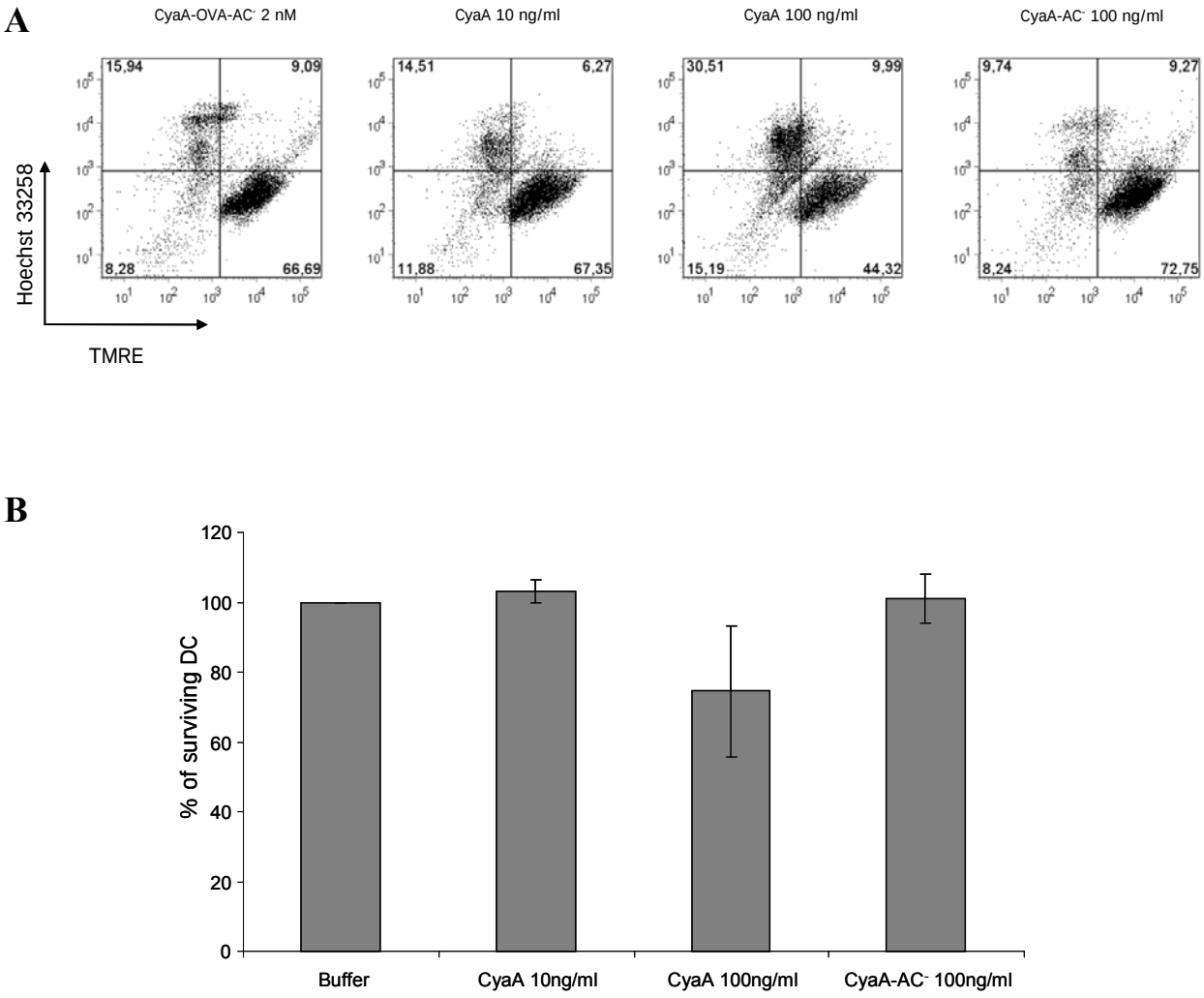

Fig. 24. Analysis of DC survival. 1x10^5/well DC were treated with 10 ng/ml or 100 ng/ml CyaA or CyaA-AC$^-$ in the presence of 2 nM CyaA-OVA-AC$^-$ for 18 hours. TMRE and Hoechst 33258 staining were used for the detection of viable cells. 10,000 cells/sample were analysed by fluorescent cytometry. Mean fluorescence intensity was calculated in the Hoechst 33258$^-$/TMRE$^+$ population, which represented live cells. Mock-treated cells were used as a negative control (Buffer). **(A)** Data are representative of 4 independent experiments. **(B)** Results are means ± SD of 4 independent experiments. The percentage of surviving cells was calculated from raw data in respect to cells incubated only with buffer (100 %).

5.1.7 Detection of DC loss after CyaA treatment

As we observed that high concentration of CyaA causes detachment of DC from plastic bottom of the well in the cultivation plate (unpublished observation), it was important to consider the possibility that CyaA-induced lack of adhesiveness of DC may lead to the cell loss and therefore to the activation of lower number of T cells. In order to exclude this aspect, LPS-treated DC were incubated with CyaA or CyaA-AC$^-$ (10 ng/ml, 100 ng/ml) in the presence of the model antigen CyaA-OVA-AC$^-$ toxoid (2 nM) for 4 hours. After the

incubation period the medium was discarded, similarly as in the antigen presentation assays before the addition of T cells (4.2.14.1). DC were fixed by PFA, stained by Hoechst 33342 and the number of attached DC per area was determined by a Laser Scanning Cytometry (4.2.14.6).

The data summarized in Fig. 25 show, that while 100 ng/ml CyaA induce the loss of DC by almost 40 % compared to mock-treated cells, CyaA in concentration 10 ng/ml did not influence the number of attached DC per area. The percentage of cells receding at the bottom of well was comparable with the number of DC treated with 10 ng/ml of CyaA-AC$^-$, an enzymatically inactive toxin.

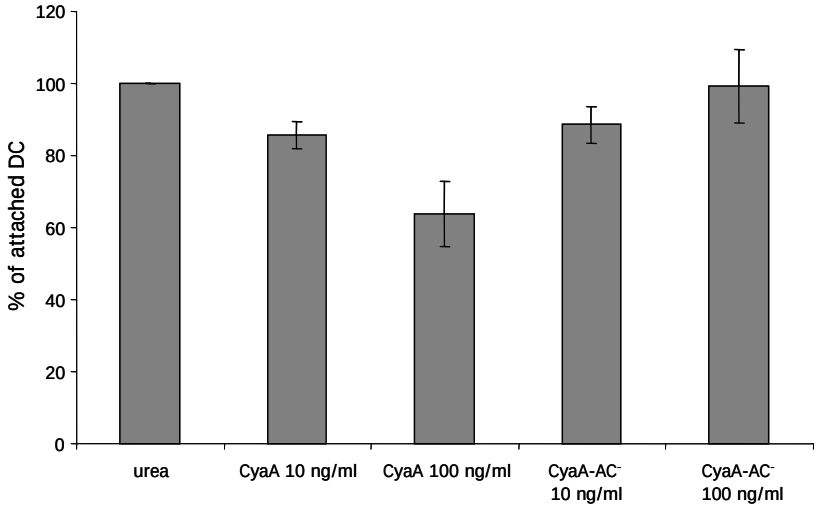

Fig. 25. Detection of DC attached to the well bottom after the treatment with CyaA or CyaA-AC$^-$ in the presence of LPS and CyaA-OVA-AC$^-$ toxoid. 1 x 10^4/well DC were treated 10 ng/ml or 100 ng/ml CyaA or CyaA-AC$^-$ in the presence of LPS and CyaA-OVA-AC$^-$ toxoid and 10 for 4 hours. Then the medium was discarded, cells were fixed by 4 % PFA and counted by laser scanning cytometry. 0.08 M urea was used as a positive control (100 %). Results are means ± SD of 3 independent experiments.

5.2 CyaA influence on Ag uptake and processing by DC

5.2.1 Analyses of CyaA effects on the uptake of OVA-FITC by DC

Since it was shown that CyaA inhibits the macropinocytosis by J774 macrophages (Kamanova et al., 2008), we wanted to investigate the possible effect of CyaA on antigen (Ag) uptake by DC as one of the possible mechanism that might account for the diminished capacity of CyaA-treated DC to stimulate T cell adaptive responses. Ovalbumin labeled with fluorescein (OVA-FITC) was used as an endocytic tracer. LPS-treated DC were incubated

with CyaA or CyaA-AC$^-$ (10 ng/ml or 100 ng/ml) at 37 ºC for 30 min, and then they were loaded with OVA-FITC (5 μg/ml) and incubated for additional 30 min. Cells incubated with OVA-FITC on ice served as a negative control for the detection of unspecific binding to membrane surface (4.2.15.1).

Considering the high expression of mannose-receptors by DC which are used during the Ag internalization (Sallusto et al., 1995), mannan was used because it competes with ovalbumin for mannose receptor and thereby blocks the mannose receptor-mediated endocytosis of ovalbumin. For determination of DC, cells were stained with anti-mouse CD11c conjugated with APC. DC were also dyed with Hoechst 33258 to exclude the dead cells from sample and analyzed by flow cytometry.

Fig. 26 demonstrates that DC treated with CyaA in concentration of 10 ng/ml engulfed the same amount of OVA-FITC as mock-treated cells suggesting that CyaA does not influence ovalbumin uptake in DC. Similarly, 100 ng/ml of CyaA did not influence the uptake of OVA. On the other hand, mannan inhibited the uptake of OVA-FITC by 70 %.

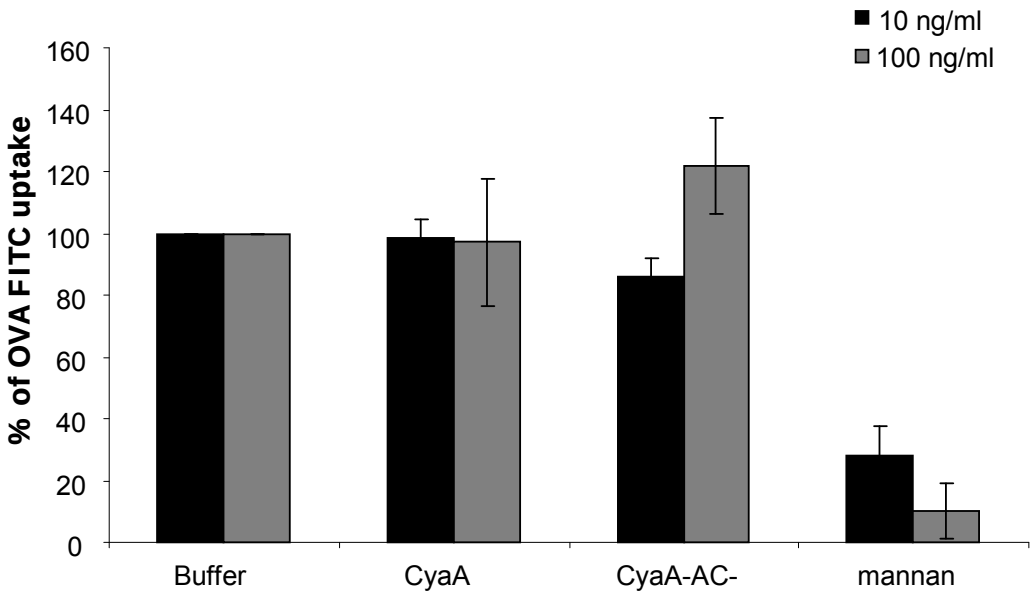

Fig. 26. Analyses of CyaA effect on the uptake of OVA-FITC by DC
1x10^5/well DC were exposed to 10 ng/ml (■) or 100 ng/ml (■) of CyaA or CyaA-AC$^-$ in the presence of LPS for 30 min and consequently loaded with OVA-FITC (5 μg/ml) for additional 30 min. For detection of CD11c$^+$ DC, mouse antibody CD11c-APC was used. 10,000 cells were analysed by fluorescence cytometry. Mannan (1 mM) was used as an inhibitor of receptor-mediated endocytosis of OVA, mock-treated cells were used as a negative control. The percentage of OVA uptake was calculated in respect to mean fluorescence intensities of cells incubated in buffer (100%). Hoechst 33258 was used to exclude the dead cells in sample. Results are means ± SD of 3 independent experiments.

5.2.2 Analysis of CyaA effects on the uptake of lucifer yellow and transferrin by DC

OVA is known to be internalized by both receptor-mediated endocytosis via mannose receptors as well as macropinocytosis (Burgdorf *et al.*, 2007). As I observed that mannan prevents the uptake of OVA by 70 %, it seems that OVA in my experimental system is probably taken mainly by receptor-mediated endocytosis (RME). As it was found in our laboratory that CyaA inhibits macropinocytosis (Kamanova et al., 2008), I decided to dissect both pathways by using transferrin as a specific marker of RME and lucifer yellow (LY) as a marker for macropinocytosis.

LPS-treated DC were treated with CyaA or CyaA-AC$^-$ (10 ng/ml or 100 ng/ml) in the presence of LPS for 30 min and consequently loaded with transferrin labeled with fluorescent Alexa 647 (10 μg/ml) or LY (500 μg/ml), respectively, for additional 30 min. Cells incubated with markers on ice served as a negative control for the detection of unspecific binding to membrane surface. Cells were stained by CD11c to detect specifically DC and to assess the viability of cells Hoechst 33258 was used. Subsequently DC were analysed by flow cytometry (4.2.15.2).

These experiments showed us that CyaA at a concentration of 10 ng/ml as well as 100 ng/ml inhibits macropinocytosis of lucifer yellow (Fig. 27A). The decrease in macropinocytosis by DCs was by ~60 % compared to mock-treated cells and cells treated with enzymaticaly inactive CyaA-AC$^-$. On the other hand, CyaA at both concentrations of 10 ng/ml and 100 ng/ml did not inhibit the transferrin-Alexa 647 uptake (Fig. 27B). These results indicate that CyaA at low concentration of 10 ng/ml strongly inhibits macropinocytosis, similarly to macrophages, whereas CyaA does not influence receptor-mediated endocytosis of transferrin.

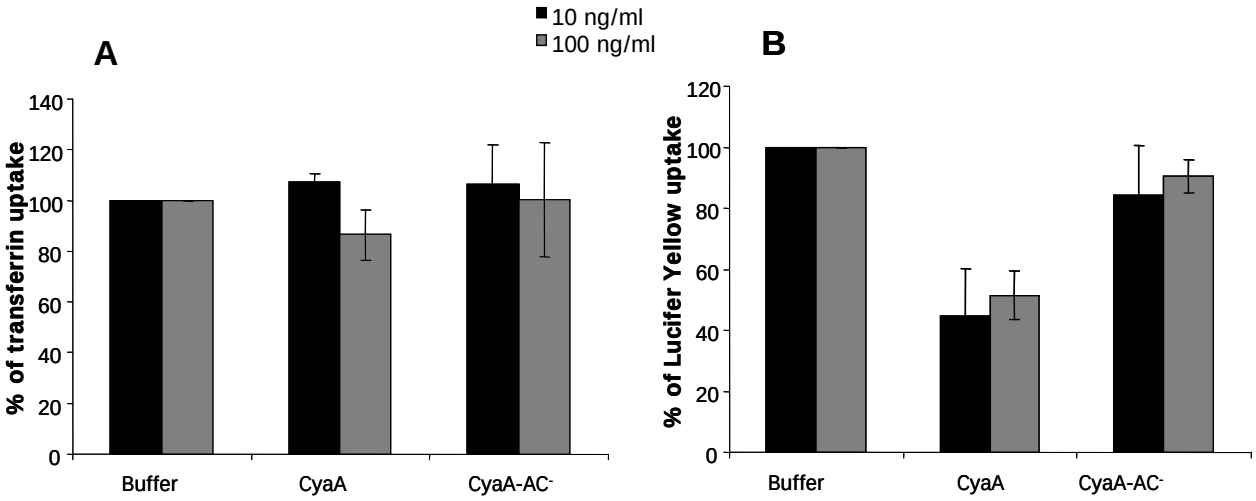

Fig. 27. Analysis of CyaA effect on the uptake of lucifer yellow and transferrin by DC
$1x10^5$ DC/well were treated with CyaA or CyaA-AC$^-$ at concentrations of 10 ng/ml (■) or 100 ng/ml (■) in the presence of LPS for 30 min. DC were then loaded with transferrin (**A**) or lucifer yellow (**B**) for further 30 min, at 37 ºC. DC were stained for CD11c (mouse antibody CD11c-APC). Hoechst 33258 was used for detection of viable cells. 10,000 cells/sample were analysed by fluorescent cytometry. The percentage of lucifer yellow or transferrin uptake was calculated in view of mean fluorescence intensities of cells incubated in buffer, which were used as a negative control. Results are means ± SD of 3 independent experiments.

5.2.3 The effect of CyaA on OVA-FITC uptake by DC treated with mannan

Since mannan inhibits OVA-FITC uptake by LPS-treated DC by 70 %, I was interested whether the remaining 30 % of ovalbumin, which were not inhibited by mannan, are taken up by macropinocytosis or whether it is due to an unspecific binding of the OVA-FITC to the DC.

As it was shown, that CyaA binding to CD11b/CD18 is inhibited in the presence of free saccharides such as D-mannose (Morova *et al.*, 2008), LPS-treated DC were firstly co-incubated with or without CyaA (10 ng/ml) for 30 min at 37 °C. Consequently mannan (1 mg/ml) was added. After 30 min at 37 °C, DC were loaded with OVA-FITC (5 µg/ml) and incubated for further 30 min at 37 °C. To determine the unspecific binding of OVA-FITC to the cell membrane, DC loaded with OVA FITC were incubated on ice. DC were stained with CD11c-APC antibody and Hoechst 33258 for detection of viable cells. 10,000 cells per sample were analysed by fluorescence cytometry (4.2.15.3).

Fig. 28 shows that mannan inhibits the OVA-FITC uptake by ~70% compared to DC treated with CyaA alone or mock-treated cells as observed in previous experiments (5.2.1.). However the addition of 10 ng/ml of CyaA did not further decrease the amount of engulfed OVA-FITC by DC, it remained at the same level as in the case of DC treated with mannan. A comparable decrease of OVA uptake was also observed by DC incubated on ice as a negative

control. From these results it seems probable, that the remaining 30 % of OVA-FITC, that were not inhibited by mannan, are due to unspecific binding of OVA-FITC to cell membrane.

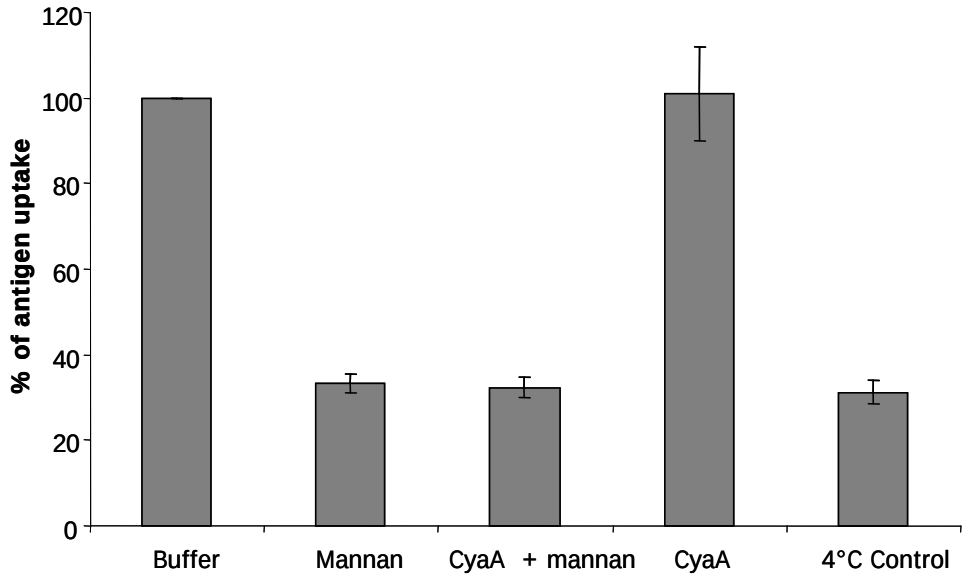

Fig. 28. Analysis of CyaA effect on OVA-FITC uptake by DC treated with mannan
$1x10^5$ DC/well were treated with or without 10 ng/ml CyaA for 30 min, then the DC were exposed to mannan for additional 30 min. Then OVA-FITC (5 μg/ml) was added for further 30 min. The endocytosis was stopped by addition of ice-cold PBS. The cells were stained for CD11c and the Hoechst 33258 staining was used to exclude the dead cells in sample. 10,000 cells/sample were analysed by fluorescent cytometry. Mock-treated cells were used as a negative control. Cells treated on ice were used to assess the background caused by unspecific binding to cell membrane. The amount of engulfed OVA-FITC was calculated with respect to negative control which stayed at 100 %. Results are means ± SD of 3 independent experiments.

5.2.4 Analysis of CyaA effects on Ag processing for MHC class II presentation in DC

It was shown that elevated cAMP in antigen presenting cells leads to antigen processing decrease (Tanaka et al., 1999, Kambayashi et al., 2001, Matousek et al., 1998). As the inhibition of antigen processing might, similarly to the inhibition of antigen uptake, lead to the decreased capacity of DC to stimulate T cell responses, we sought to investigate whether CyaA influences the Ag degradation and processing for MHC class II molecules in DC. For that purpose LPS-treated DC were pre-incubated with CyaA or CyaA-AC^- (10 ng/ml, 100 ng/ml), or chloroquine for 30 min and subsequently loaded with a mixture of two fluorescent ovalbumines. Ovalbumin labeled with Alexa 647 (OVA-Alexa 647) was used as a marker of antigen uptake and ovalbumin labeled with BODIPY®FL dye (OVA-DQ BODIPY™) ,which emits the fluorescence only when proteolytically degraded, was used as a marker of Ag

uptake and processing. Chloroquine was used as a positive control because of its ability to increase endosomal pH, thereby disrupting the Ag degradation and processing (4.2.15.3).

$1x10^6$ DC per a microtube were treated with the reagents described above at 37 ºC. Every 10 minutes, $1x10^5$ cells per one sample were transferred into ice-cold PBS + 2 % FCS to stop the endocytosis. The cells were consequently stained for CD11c and prior the measurement by fluorescence cytometry, Hoechst 33258 was added to exclude the dead cells in samples.

The time course analysis is shown in Fig. 29A and B. The representative experiments demonstrate the amount of taken and processed OVA during 40 minutes. During the first 30 min, the amount of engulfed and processed OVA had risen rapidly and then stagnated. The experimental scheme and the data evaluation using FlowJo software are described in methods (Fig. 16B)

Since most of the OVA is taken and degraded within 30 minutes, the data were evaluated after the time point of 30 min. The ratio of mean fluorescence intensity percentage of OVA-DQ to OVA-Alexa 647 was calculated to assess the degree of degraded OVA in DC (Fig. 16C in methods). The results are summarized by Fig. 29C and Tab. 4. Even though it seems from the means or ratios of OVA-DQ/OVA-Alexa 647, that CyaA at concentration 10 ng/ml inhibits the processing of antigen compared to mock-treated cells; it is not significant with respect to standard deviations. CyaA at the concentration of 10 ng/ml does not influence the processing of OVA. Interestingly, 100 ng/ml of CyaA raised the rate of processed OVA by ~20%, in comparison to mock treated cells suggesting that CyaA might even accelerate the Ag processing in DC.

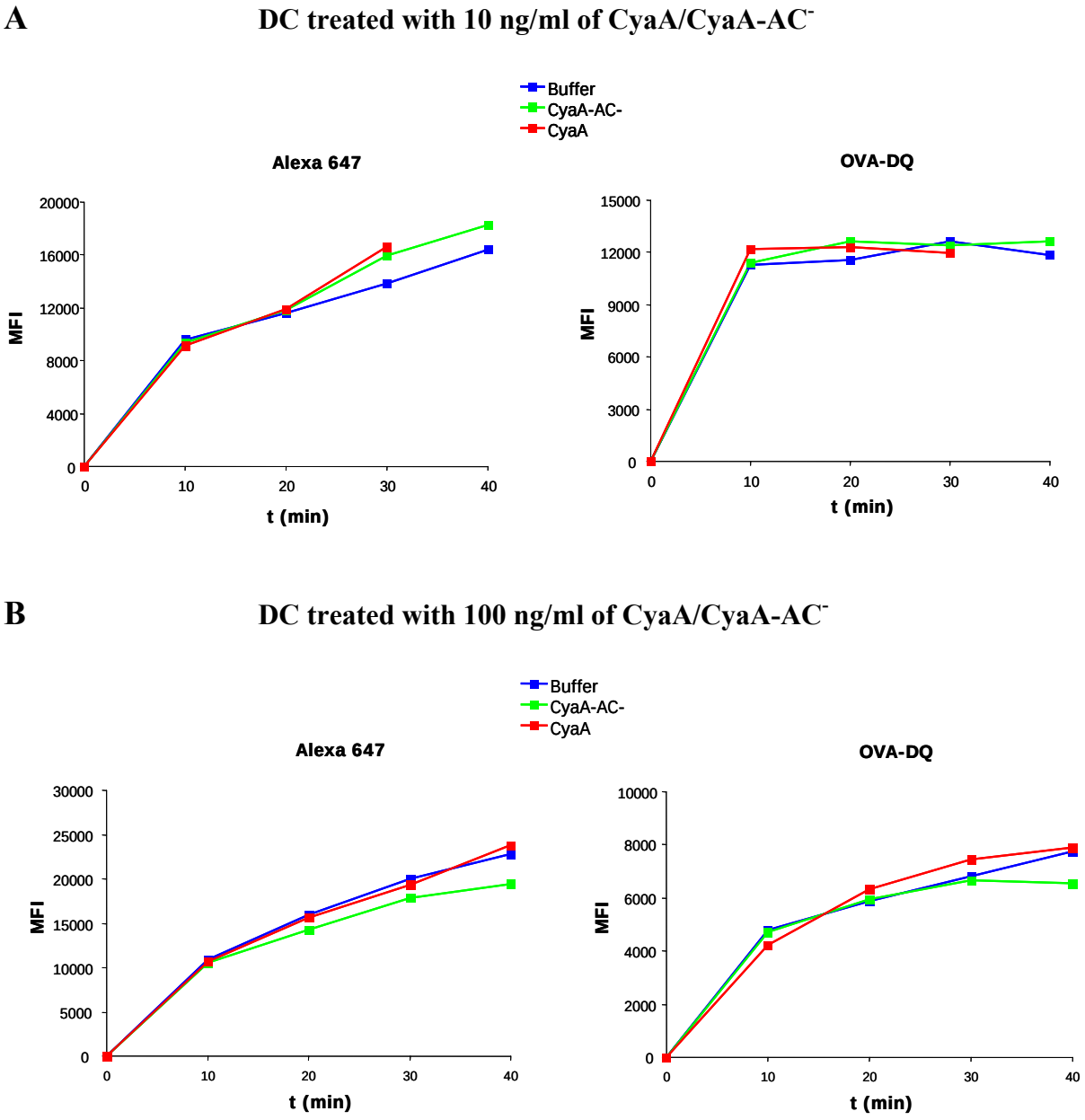

Fig. 29. Time course analysis of CyaA effect on the OVA uptake and processing
DC were treated with 10 ng/ml **(A)** or 100 ng/ml **(B)**CyaA or CyaA-AC$^-$ for 30 min and consequently a mixture of two fluorescently labeled ovalbumins (OVA-Alexa 647, OVA-DQ BODIPY^{TM}) was added for further 30 min. Samples were collected over time (40 min). MFI of cells treated with CyaA – red curve (■), CyaA-AC$^-$ - green curve (■), Buffer – blue curve (■). Data are representative from 3 independent experiments.

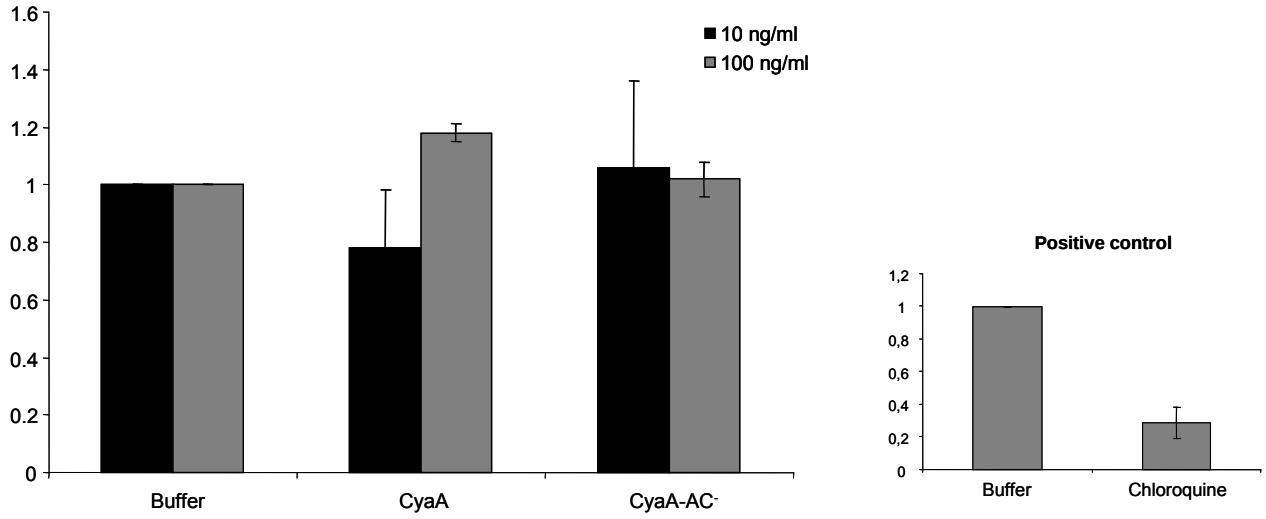

Fig. 29C. CyaA effect on OVA processing in DC. The ratio of % MFI OVA-DQ/% MFI OVA-Alexa 647 was calculated (Fig. 16 C in methods). DC were treated with 10 ng/ml (■) or 100 ng/ml (■) CyaA or CyaA-AC$^-$. Mock-treated cells were used as a negative control (100 %). Chroroquine was used as a positive control. Results are means ± SD of 4 independent experiments.

Tab. 4. The ratio % MFI OVA-DQ / % MFI Alexa 647

	10 ng/ml	**100 ng/ml**
Buffer	1.0 ± 0.0	1.0 ± 0.00
CyaA	0.78 ± 0.2	1.18 ± 0.03
CyaA-AC$^-$	1.06 ± 0.3	1.02 ± 0.06

5.2.5 Analysis of CyaA effect on the proteasome proteolytic functions of DC

Since CyaA hampers the capacity of LPS-stimulated DC to prime B3Z T cell hybridoma as well as to stimulate proliferation of primary murine CD8$^+$ T cells (Adkins et al., prepared for submission), I investigated whether CyaA might influence proteasome proteolytic functions, thereby the antigen processing for MHC class I molecules in DC.

In order to find out whether CyaA affects proteasome proteolytic functions, LPS-treated DC were incubated with CyaA, CyaA-AC$^-$ (10 ng/ml, 100 ng/ml) or 10 μM lactacystin for 30 min or 1 hour, respectively, at 37 ºC. The cells were lyzed and 50 μg of proteins were mixed with fluorescent peptide substrates with inserted AMC (7-amino-4-methylcoumarin) (4.2.15.4), which do not emit fluorescence until they are cleaved by a specific proteasome subunit. The fluorescence of loosen AMC was assessed by a fluoreader ($380_{ex}/460_{em}$). Lactacystin was used as a positive control due to its ability to inhibit poteasomal processing functions (Fenteany & Schreiber, 1998).

Fig. 30 shows that CyaA at a concentration of 10 ng/ml did affect neither chymotrypsin-like, trypsin-like nor caspase-like proteasome proteolytical functions, compared

to both, the mock-treated cells and cells treated with an enzymatically inactive CyaA-AC$^-$. However, 100 ng/ml of CyaA increased the amount of degraded protein by 20 % by caspase-like and chymotrypsin-like activity but not by trypsin-like activity suggesting that CyaA at higher concentration might accelerate the proteasomal processing.

Lactacystin inhibited only chymotrypsin-like activity but not trypsin-like and caspase-like activity in our experiments which might be in agreement with the inhibitory effects of lactacystin that was shown to inhibit only trypsin-like and chymotrypsin-like proteasom activities irreversibly and all activities were decreased at different rates (Fenteany et al., 1995)

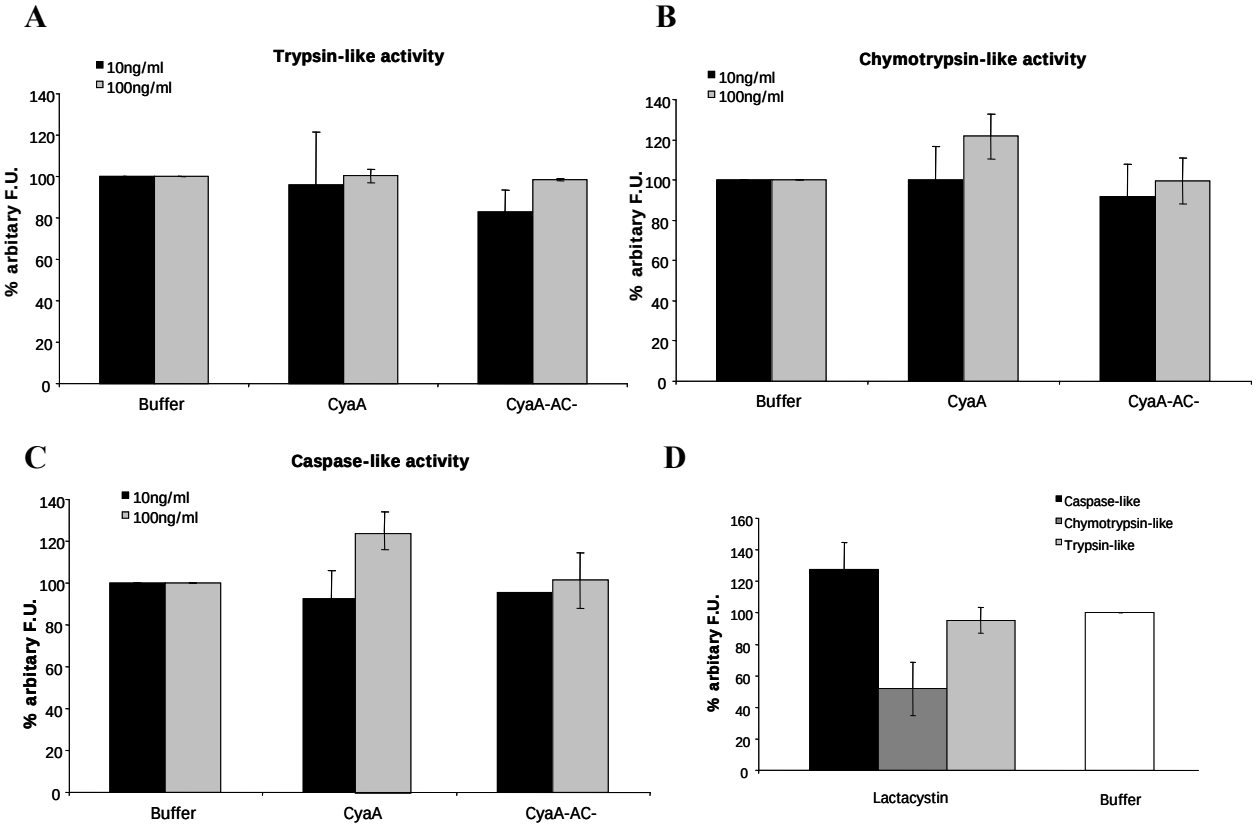

Fig. 30. The effect of CyaA on Ag processing for MHC class I presentation
1x10^6 DC/well were treated with 10 ng/ml (■) or 100 ng/ml (■) CyaA, CyaA-AC$^-$ or 10 μM lactacystin. Then DC were lyzed and fluorescent peptide substrates were added to the 50 μg of cellular proteins. The emitted fluorescence was measured using a fluororeader ($380_{ex}/460_{em}$). Mock-treated cells were used as a negative control. Results are means ± SD of 4 independent experiments. (A) Z-Leu-Leu-Glu-AMC fluorogenic substrate was used to detect caspase-like activity of proteasome, (B) Suc-Leu-Leu-Val-Tyr-AMC fluorogenic substrate was used to detect chymotrypsin-like activity of proteasome, (C) Boc-Leu-Arg-Arg-AMC or Ac-Arg-Leu-Arg-AMC fluorogenic substrate was used to detect trypsin-like activity of proteasome at concentration of 100 μM.

6. DISCUSSION

CyaA binds to myeloid cells via CD11b/CD18 and translocates its adenylate cyclase (AC) domain into the cell cytosol where it catalyzes the conversion of ATP molecules to cAMP, thereby affecting various physiological functions of the cells. It was shown that CyaA decreases the monocyte driven $CD4^+$ T cell proliferation which was dependent on the AC activity (Boschwitz et al., 1997). Indeed, cAMP-elevating agents incubated with DC also inhibited $CD4^+$ T cell proliferation (Kambayashi et al., 2001). In our laboratory, we observed that CyaA-treated DC are inefficient to present soluble ovalbumin and to stimulate the IL-2 production by $CD8^+$ and $CD4^+$ T cell lines as well as the proliferation of primary ovalbumin-specific T cells (Adkins et al., prepared for submission). On the basis of these data, the aim of my work was to investigate whether CyaA incubated with DC is able to inhibit presentation of another model antigen, CyaA-OVA-AC^- toxoid carrying MHC class I and II-specific OVA epitopes. Moreover, the possibility that CyaA inhibits the capacity of DC to internalize and process antigens was investigated.

Since recombinant genetically detoxified CyaA was shown to be a useful delivery tool for various antigens into cell cytosol that were consequently processed and presented to T cells (Simsova et al., 2004), CyaA-AC^- toxoid (CyaA-OVA-AC^-) carrying three OVA epitopes, as a model antigen, was constructed. We showed that the constructed CyaA-OVA-AC^- toxoid is efficiently presented to specific $CD4^+$ as well as $CD8^+$ T cell hybridomas at 1 nM and 0,5 nM concentration, respectively. Surprisingly, in the case of MHC class II presentation, increased levels of IL-2 above the background levels in supernatants were detected when cells were incubated with an empty negative control CyaA-AC^- w/o OVA in concentration over 10 nM. Since LPS and other bacterial stimuli are able to induce IL-2 production in DC (Granucci *et al.*, 2003), a possible explanation could be that residual LPS in a sample of CyaA-AC^- w/o OVA may have stimulated DC to produce IL-2. Moreover, it is not clear if the toxoid itself is capable of inducing IL-2 production in DC at higher concentrations which was shown to stimulate T cells. It would be tentative to speculate about such activity of the toxoid as detoxified CyaA-AC^- carrying antigenic epitopes is an effective delivery tool without the need of any additional adjuvant (Dadaglio *et al.*, 2000).

More importantly, the results showed that wild type CyaA toxin, already in concentration of 1 ng/ml, inhibited the capacity of LPS-treated DC to present CyaA-OVA-AC^-, thereby decreasing IL-2 production in $CD4^+$ as well as $CD8^+$ T cell hybridomas, which was dependent on the increased levels of cAMP generated by CyaA. The effect of elevated cAMP due to AC activity of CyaA was confirmed by db-cAMP, which hampered the IL-2

production in both T cell hybridomas. These observations correlate well with the data obtained by Adkins and co-workers on the presentation of soluble ovalbumin (Adkins et al., prepared for submission) suggesting that CyaA, indeed, inhibits the T cell stimulatory capacity of LPS-treated DC.

DC treatment with LPS and CyaA might have caused some unwanted effects, like cell death, or cell detachment from the cultivation plate, which might have an account for the inhibition of $CD4^+$ as well as $CD8^+$ T cell response. Since, CyaA was shown to be cytotoxic (Gueirard et al., 1998, Khelef et al., 1993), it was necessary to rule out the possibility that DC death and subsequent lesser number of DC-T-cell contacts could have caused the decrease in IL-2 production by T cell hybridomas. Flow cytometry analyses showed that CyaA at concentration of 10 ng/ml did not decrease the number of viable DC treated with LPS and CyaA-OVA-AC^- toxoid. Further, it was ruled out that the impairment of DC capacity to stimulate T cell response would be due to CyaA-mediated loss of cellular adhesion that could have consequently led to the loss of DC during the experiment and the decrease in T cell stimulation. There is also the possibility that T cells itself could have been affected directly by CyaA as it was observed that CyaA caused inhibition of T cell proliferation when added to the T cells (Paccani et al., 2008, Rossi Paccani et al., 2009). As T cells do not express CD11b/CD18 integrin on their surface, a much higher concentration of CyaA was used to influence T cells. Since very low CyaA concentration was used together with an extensive washing step in our experiments, it seems very unlikely that T cells were affected directly by Cya in our study.

The inhibition of antigen uptake and processing for the MHC class I and II-mediated presentation by DC represents an important strategy of some bacterial pathogens like *Helicobacter pylori, Chlamydia trachomatis* or *Mycobacterium tuberculosis*. (Molinari et al., 1998, Zhong et al., 1999, Hmama et al., 1998) to affect T cell adaptive responses. In our laboratory we observed that CyaA strongly inhibits macropinocytosis in macrophages (Kamanova et al., 2008). Therefore, in the second part of my work, the possible CyaA effects on antigen uptake and processing for the presentation on MHC class I and II molecules were investigated. Flow cytometry analyses showed that CyaA, even at concentrations of 100 ng/ml does not influence uptake of OVA-FITC and fluorescently labelled transferrin, a marker for receptor-mediated endocytosis suggesting that CyaA does not affect this antigen uptake pathway. Furthermore, it seems that OVA at concentration of 5 μg/ml was taken up almost exclusively by receptor-mediated endocytosis, and not by macropinocytosis (Adkins et al., prepared for submission) as CyaA-treatment did not further decrease OVA uptake in mannan-treated cells. On the other hand, CyaA inhibited the uptake of lucifer yellow by

macropinocytosis by approximately 60 % which is in agreement with the findings of Kamanova et al. (2008) in macrophages (Kamanova et al., 2008). These results suggest that the decreased presentation of soluble ovalbumin by CyaA in LPS-treated DC observed by Adkins et al. is not primarily due to the inhibition of antigen uptake. However, it cannot be excluded that the presentation of antigens taken up predominantly by macropinocytosis might contribute to the inhibition of T cell stimulatory capacity of the DC by CyaA.

The impairment of antigen processing for MHC class I and II-restricted presentation by CyaA could be another possible explanation for the impairment of antigen presentation to T cells by LPS stimulated DC. However, in this study CyaA at concentration of 10 ng/ml did not influence the antigen processing of ovalbumin in LPS-treated DC for MHC class II-restricted presentation. Furthermore, 100 ng/ml even modestly increased the degree of ovalbumin processing. The study of Bimczok et al., (2007) also showed that cholera toxin, another cAMP-elevating toxin, did not affect OVA-DQ processing in porcine DC (Bimczok *et al.*, 2007). On the other hand, Tanaka et al. (1999) observed a decrease of intracellular degradation of ovalbumin (OVA-DQ) by B lymphoma cells treated with cholera toxin (Tanaka et al., 1999). However, in both cases different toxin and cells were used which might not reflect the action of CyaA. Similarly, CyaA did not influence the proteasome proteolytic functions at concentration 10 ng/ml and even slightly increased chymotrypsin-like and caspase-like activity of the proteasome at higher concentration of 100 ng/ml. Indeed, the enhancement of chymotrypsin-like activity was observed, when NRK (normal rat kidney) cells were treated with forskolin, which elevates cAMP in cells (Zhang et al., 2007). However, the enhancement of the trypsin-like activity of the proteasome, which was not observed in this study, was also described (Zhang et al., 2007). On the other hand, our results do not correlate with data of Hoang et al. (2004) who described an inhibitory effect of 8-CPT-cAMP (8-Bromoadenosine cAMP), an analogue of cAMP and some cAMP-elevating agents such as forskolin and IBMX on proteolytic proteasome functions in MSC-1 cells, mouse Sertoli cells (Hoang et al., 2004). Furthermore, it cannot be also excluded that the increased OVA processing for MHC class I and II, observed when high concentration of 100 ng/ml of CyaA was used, might actually lead to the degradation of the protein antigen without the generation of immunodominant epitopes. This hypothesis remains to be further tested.

Taken together, this study showed that CyaA decreases the capacity of LPS-treated DC to stimulate $CD4^+$ as well as $CD8^+$ T cell response to CyaA-OVA-AC^- as a model antigen. Furthermore, it was excluded that CyaA affects receptor-mediated endocytosis and antigen processing for MHC class I and II presentation. CyaA modulates DC maturation and cytokine production induced by LPS therefore it seems conceivable that one of the factors of

impairment of T cell response is the decrease in expression of co-stimulatory molecules CD40, CD86 and CD54 (Skinner et al., 2004, Ross et al., 2004, Boyd et al., 2005), which are necessary for activation of T cells. Another factor may be altered spectrum of produced cytokines leading to the suppression of TNF-α and IL-12 and the enhancement of IL-10 production, which was documented to be inhibitory for T-cell proliferation and IL-2 production (Taga & Tosato, 1992). Furthermore, it was shown that bone marrow macrophages infected with *B. bronchiseptica* produced prostaglandin E2, which has negative effects on antigen-induced $CD4^+$ T cell proliferation (Siciliano et al., 2006) This suggests that other factors produced by DC upon CyaA treatment might play role in the inhibition of T cells.

B. pertussis infection was shown to be eradicated by $CD4^+$ Th1 (Mills et al., 1993) cells in *in vivo* studies. However, antigen specific Th1 responses in the lung and local lymph nodes are severely suppressed during the acute phase of the infection (Mills et al., 1993). We can speculate that CyaA effects on DC capacity to stimulate T cells are in favour to local *B. pertussis* infection. Moreover, CyaA might co-operate with other virulence factors, such as filamentous hemagglutinin (FHA) in hampering lung T cell proliferation, which was shown to be partly due to lack of costimulatory molecule CD28 (McGuirk *et al.*, 1998). Furthermore, FHA, similarly to CyaA, stimulates IL-10 production by DC and it was shown to be responsible for the induction of pathogen-specific T regulatory 1 (Tr1) cells upon infection (McGuirk & Mills, 2002). These cells further produce IL-10 and expand the suppression of Th1 response. These negative effects on T cell responses at the beginning of the *B. pertussis* infection can be considered to be harmful especially for immunodeficient individuals, such as people with AIDS or infants with not perfectly developed immunity.

The role of $CD8^+$ T cells in *B. pertussis* infection has not been completely dissected yet. According to study by Mills et al. (1993), the adoptive transfer of $CD8^+$ T cells did not confer any protection in mice challenged by respiratory *B. pertussis* (Mills et al., 1993). However, CyaA-treated DC were shown to hamper $CD8^+$ T cells expansion *in vivo* (Adkins et al., prepared for submission), which correlates with the *in vitro* results obtained herein. Therefore it cannot be excluded that these cells participate in immune responses against *B. pertussis*, especially by their ability to produce IFNγ, as it was shown that exhibition of mice with IFNγR defect to *B.pertussis* infection caused an atypical disseminated disease (Skerry *et al.*, 2009, Mahon *et al.*, 1997). In addition, $CD8^+$ T cells can also clear the organism of infected cells by intracellular pathogens, such as *Listeria monocytogenes* or *Chlamydia pneumoniae* (Tvinnereim & Wizel, 2007, Tvinnereim & Harty, 2000). Even though *B.pertussis* is supposed to be an extracellular pathogen, there is a number of studies claiming

that *B.pertussis* is not only able to survive intracellularly in human macrophages (Hellwig *et al.*, 1999) but it is also able to replicate in nonacidic compartments with characteristics of early endosomes (Lamberti *et al.*, 2010). We may speculate that $CD8^+$ T cells primed by antigens of *B. pertussis* can possibly eliminate such macrophages representing an intracellular niche for *B. pertussis*. The decreased $CD8^+$ T cell proliferation and expansion due to impaired DC capacity by CyaA effect might hence contribute to maintenance of *B. pertussis* in alveolar macrophages and to infection spreading.

7. SUMMARY

1) A model antigen, CyaA-OVA-AC$^-$ toxoid carrying OVA epitopes specific for CD8$^+$ and CD4$^+$ T cell hybridomas, was successfully constructed and purified. It was shown that the CyaA-OVA-AC$^-$ toxoid delivers OVA epitopes into DC for presentation to specific CD8$^+$ and CD4$^+$ T cell hybridomas.

2) It was shown that enzymatically active CyaA toxin at the concentration of 10 ng/ml inhibits the presentation of OVA epitopes carried by CyaA-OVA-AC$^-$ toxoid on MHC class I and II molecules as determined by a decreased IL-2 production of CD8$^+$ and CD4$^+$ T cell hybridomas.

3) It was excluded that the CyaA-mediated inhibition of DC capacity to present OVA epitopes carried by CyaA-OVA-AC$^-$ toxoid might have been caused by death of cells induced by the 10 ng/ml of CyaA toxin. Similarly it was shown, that CyaA at this concentration had no effect on cell adhesivity to the well bottom that did not cause loss of cells and of DC-T cell contacts.

4) CyaA did not affect OVA or trasferrin uptake via receptor-mediated endocytosis in any of the used toxin concentrations (10 ng/ml and 100 ng/ml). Moreover, in our study it was observed that OVA at the concentration of 5 μg/ml is taken up only by receptor-mediated endocytosis in DC. On the other hand, CyaA strongly inhibited macropinocytosis of lucifer yellow by (~70 %) already at the concentration of 10 ng/ml.

5) CyaA at concentration of 10 ng/ml did not affect OVA processing by DC for MHC class II-restricted presentation. In fact, CyaA at concentration of 100 ng/ml modestly increased the amount of processed OVA by DC.

6) CyaA at concentration of 10 ng/ml did not influence any of the described proteasome proteolytic activities. However, CyaA at the concentration of 100 ng/ml even modestly increased caspase-like and chymotrypsin-like activity of proteasome.

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