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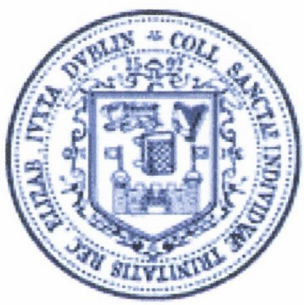
Modulation of innate and adaptive immune responses by adenylate cyclase toxin from *Bordetella pertussis*

Pádraig John Ross

A thesis submitted to the University of Dublin Trinity College
for the degree of
Doctor in Philosophy



October 2005

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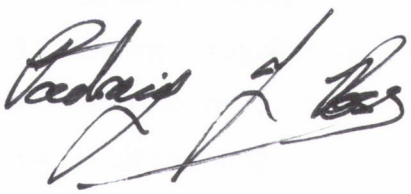
Signed: 

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Appendix I **i**

Declaration of Authorship

This thesis has not been previously submitted in whole or in part to this or any other university for any other degree. This thesis represents the sole work of the author with the exception of work presented in chapter 5, which was done in collaboration with Dr. Aoife Boyd.

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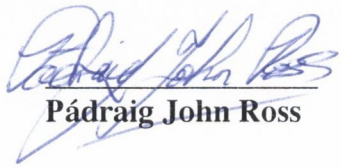

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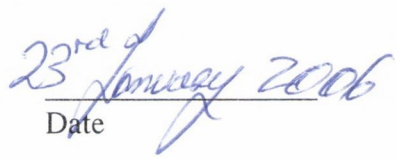

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Publications

Boyd AP.*, Ross PJ.*, Conroy H., Mahon N., Lavelle EC. and Mills KH. (2005) *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. *Journal of Immunology* 15, 175, 730-738.
*Joint authorship

Ross PJ., Lavelle EC., Mills KHG. and Boyd AP. (2004) Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infection and Immunity* 72, 3, 1587-1579.

Ross, PJ., Boyd, AP., Conroy, H., Lavelle, EC. and Mills, KHG. (2004) Regulation of innate and adaptive immunity by a non-toxic adenylate cyclase toxin from *Bordetella pertussis. Immunology* 113, s1, 17-19.

Abstract.

CyaA belongs to the repeat-in-toxin (RTX) family of pore-forming toxins that require posttranslational acylation to lyse eukaryotic cells. While it was known that CyaA binds to a variety of cell types, catalyses the generation of intracellular cAMP and inhibits phagocytic cell functions, the influence of CyaA on the immune response was not fully understood. In this study, the modulation of innate and adaptive immune responses by CyaA was investigated. The study of pathogen-derived molecules is complicated by co-purification of molecules that are themselves immunomodulatory. Similar to RTX toxins the hydrophobic nature of CyaA results in tight binding of lipopolysaccharide (LPS) making it difficult to purify CyaA free of LPS.

In this thesis the adjuvant and immunomodulatory properties of CyaA were examined and the possible contribution of LPS, known to be present in purified CyaA preparations was assessed. It was demonstrated that CyaA enhanced antigen-specific interleukin-5 (IL-5) and IL-10 production and immunoglobulin (Ig) G1 antibodies to co-administered antigen *in vivo*. Antigen-specific $CD4^+$ T-cell clones generated from mice immunised with antigen and CyaA had cytokine profiles characteristic of Th2 or type 1 regulatory T (Tr1) cells. In addition, nasal delivery of CyaA induced Th2 and Tr1 cells and antigen-specific antibodies and promoted the induction of antigen-specific IgA in the lung. Since innate immune cells direct the induction of T-cell subtypes and considering that CyaA can target innate immune cells by binding surface expressed CD11b/CD18 the influence of CyaA on the activation of dendritic cells (DC) and macrophages was examined. CyaA significantly augmented LPS-induced IL-6 and IL-10 and inhibited LPS-driven TNF-α, IL-12p70 and MIP-1α production from bone marrow-derived DC and macrophages. CyaA also enhanced cell surface expression of CD80, CD86, and MHC class II on immature DC. CyaA modulated CpG-induced responses from innate cells in a similar manner. This study also indicated that many of the biological activities of CyaA reported previously were complicated by contaminating LPS. However, the results here clearly demonstrate that CyaA synergises with TLR agonists to promote IL-10 production from innate immune cells but that CyaA can also upregulate CD80 and downregulate basal expression of the surface co-stimulatory molecules CD40 and ICAM-I on DC. CyaA also suppressed the transcription of pro-inflammatory genes and upregulated genes associated with anti-inflammatory responses. These findings demonstrate that while CyaA modulates gene transcription, the activation of innate cells *in vitro* by CyaA is dependent on a second signal through a TLR and that CyaA can promote Th2/Tr1-cell responses by inhibiting IL-12p70 and promoting IL-10 production by DC and macrophages.

Posttranslational acylation of CyaA by the specific accessory protein CyaC was thought to facilitate cell binding, pore formation and the translocation of the enzymatic domain into the cytosol of target cells where it catalyses the formation of supraphysiological levels of cAMP. The roles of acylation and enzymatic activity in the immunomodulatory and lytic effects of CyaA were examined. It was found that the adenylate cyclase activity of CyaA was necessary for its modulatory effects on murine innate immune cells. In contrast, acylation was not essential for the accumulation of cAMP or the immunomodulatory function of CyaA, but was required for maximal caspase-3 activation and cytotoxic activity. Furthermore, enzymatic activity was required for the induction of Th2 and Tr1 cells *in vivo,* while acylation was not necessary for adjuvanticity. These findings demonstrate that the enzymatic activity plays a critical role in the immunomodulatory effects of CyaA, whereas acylation facilitates the induction of apoptosis and cell lysis

The results in this thesis have elucidated the mechanism of immunomodulation by CyaA and demonstrated that CyaA promotes the induction of anti-inflammatory and regulatory innate and adaptive immune responses.

Abbreviations

3,3′,5,5′-Tetramethylbenzidine	TMB
acyl carrier protein	ACP
Acylated CyaA	A-CyaA
Adenosine triphosphate	ATP
Adenylate cyclase toxin	CyaA
a-hemolysin	HlyA
Antigen presenting cells	APC
Arginine	Arg
Asparagine	Asn
Bone marrow-derived dendritic cells	BMDC
Calf intestinal alkaline phosphatase	CIAP
Calmodulin	CaM
Centre of differentiation	CD
Cholera toxin	CT
Complement receptor-3	CR3
CpG containing oligodeoxynucleotides	CpG-ODN
Cyclic adenosine mono-phosphate	cAMP
Cytotoxic T lymphocytes	CTL
Dendritic cells	DC
Deoxyribonucleic acid	DNA
Dermonecrotic toxin	DNT
Diethylaminoethyl	DEAE
Dipteria, tetanus, pertussis	DTP
Dulbeccos modified eagles media	DMEM
Epstein-Barr Virus	EBV
Filamentous haemagglutinin	FHA
Fimbriae	FIM
Fluorescence-activated cell sorting	FACS
Foetal calf serum	FCS
Glutamic acid	Glu
Glutamine	Gln
Graft versus host	GVH
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Hepatitis C Virus	HCV
Histidine	His
Histidine at position 106	His^{106}
Histidine at position 63 mutated to an Alanine residue.	H63A
Histidine at position 63 mutated to an Alanine residue.	His^{63} ->Arg
IL-1RI-associated kinase	IRAK

Immature DC	iDC
Immunoglobulin	Ig
Immunopotentiating reconstituted influenza virosomes	iriv
Inactive adenylate cyclase CyaA	iAC-CyaA
Intercellular adhesion molecule	ICAM
Interferon regulatory factor	IRF
Interleukin	IL
Keyhole limpet hemocyanin	KLH
Leucine	Leu
Leukocyte function-associated antigen	LFA
Lipopolysaccharide	LPS
LPS binding protein	LBP
Luria Bertani broth	LB
Lymphocytic choriomeningitis virus	LCMV
Lymulus amoebocyte lysate	LAL
Major histocompatibility complex	MHC
Methionine	Met
MyD88 adaptor-like	MAL
Natural killer	NK
Non-acylated CyaA	NA-CyaA
o-phenylendiamine	OPD
Pattern recognition receptor	PRR
PBS, BSA, Azide	PBA
Pertactin	PTN
Pertussis toxin	PT
Phosphate buffered saline	PBS
Phosphorothioate- stabilized oligodeoxynucleotide-containing CpG motifs	CpG-ODN
Polymerase chain reaction	PCR
Red blood cells	rbc
Repeat-in-toxin	RTX
Respiratory syncytial virus	RSV
Ribonucleic acid	RNA
Roswell Park Memorial Institute	RPMI
Serine	Ser
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	SDS-PAGE
T cell receptor	TCR
N,N,N',N'-Tetramethylethylenediamine	Temed
T helper	Th
TIR-domain-containing adaptor protein	TIRAP
TIR-domain-containing adaptor-inducing interferon-β	TRIF
Toll-IL-1 receptor-resistance	TIR

Toll-like receptor	TLR
Tracheal cytotoxin	TCT
TRIF related adaptor molecule	TRAM
Tris, Boric acid, EDTA	TBE
Tryptophan	Trp
tumor necrosis factor receptor-associated factor 6	TRAF6
Type 1 regulatory T cell	Tr1
Valine	Val

Chapter 1

INTRODUCTION

1.1 General Introduction

Adenylate cyclase toxin (CyaA) is an important virulence factor produced by *Bordetella pertussis*, the causative agent of whooping cough and is required for successful infection. CyaA causes lysis and cell death in a variety of cells, including immune cells, but while the structure of the protein has been described, the modulation of immunity by CyaA had not been fully elucidated (Friedman *et al.*, 1987, Ladant and Ullmann, 1999). The primary objective of this research was to study how CyaA modulates both the innate and adaptive immune responses and to establish how its immunomodulatory activity might play a role in the pathogenesis of *B. pertussis*. In this project traditional immunological approaches were employed to investigate if CyaA could modulate innate and adaptive immunity and what type of immune response was induced by CyaA.

In addition to CyaA causing lysis and cytotoxicity of host cells, CyaA also binds to cells of the immune system and perturbs immune responses required for bacterial clearance (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987). Like CyaA, filamentous haemagglutinin (FHA), an important adhesin required for *B. pertussis* infection, binds to cells expressing CD11b/CD18 and consequently may modulate the innate immune responses (Guermonprez *et al.*, 2001, McGuirk *et al.*, 2002). Pertussis toxin (PT) expressed by *B. pertussis* and cholera toxin (CT) expressed by *Vibrio cholerae* bind cells of the innate immune system and exerts activities that result in an increase in cyclic adenosine mono-phosphate (cAMP). PT exerts this effect by inactivating the Gi protein that normally regulates cAMP production, while CT permanently activates the host cells adenylate cyclase. This increase in intracellular cAMP results in modulation of the immune responses (Cropley *et al.*, 1995, Lavelle *et al.*, 2004). CyaA also catalyses the formation of cAMP in target cells. It was therefore of importance, to understand how CyaA could modulate the immune system and to determine what role the enzymatic and cell binding activities of CyaA play in this immunomodulation. To this end, mutants of CyaA with amino acid substitutions in domains critical to the induction of cAMP and altered interaction with target cells were constructed and used to investigate how these activities contributed to the modulation of innate and adaptive immunity by CyaA. In addition, microarray technology was used

to determine the global effect of CyaA on cells of the innate immune system. This technique allows for simultaneous analysis of the entire transcriptome and allowed the activation of the innate immune system by CyaA to be investigated in great detail. The data generated identified key events in the activation of the immune system. As well as furthering the knowledge of how CyaA interacts with the immune system and plays a role in immunity to *B. pertussis* infection, it may help to expand the understanding of how the immune system is activated and modulated and may help to identify potential targets for the therapy of infections and immune mediated disorders.

Pathogens such as bacteria, fungi and helminth parasites express a variety of toxins, virulence factors and structural components that are essential for the survival and proliferation of the organism in its host. Many of these toxins have been characterised and shown to play a role in subverting the host immune response against the pathogen. Many of these molecules have immunomodulatory activities, that may also have potential use as therapeutic agents against immune mediated diseases. The application of pathogen-derived molecules as therapeutic agents for immune mediated diseases is dependent on a thorough understanding of their mechanism of action. Therefore the knowledge of how CyaA modulates innate and adaptive immunity may assist in its development as a potential immunotherapeutic molecule.

1.2 Immunity

Protection against infection is vital to the survival of all animals. Immunity to disease may be defined as the body's ability to mount an effective defence against a pathogen to prevent it infiltrating, proliferating and causing damage and disease in the host. However, if immune responses are not tightly controlled they can result in severe inflammation and collateral tissue damage.

1.2.1 Vaccination

Induction of immunity against infectious diseases in susceptible individuals by vaccination has been a goal of scientists since the 18th century. Researchers began by using killed or attenuated/crippled pathogens to induce protective immunity. Although the techniques would be considered crude by today's standards, they did succeed in the

elimination of smallpox. A dramatic reduction in diseases such as polio, whooping cough, rabies and tuberculosis was also achieved using similar techniques. However, certain of the successes were limited to areas of the world where vaccination programs are widespread. The success of vaccination and the development of antibiotics led many to believe that all infectious diseases could be conquered. However, the evolution of antibiotic resistant bacteria and the emergence of new pathogens necessitate the continuing development and improvement of vaccination strategies.

Whole live or attenuated vaccines have been very successful at protecting against many infectious diseases. However, these vaccines do retain residual toxicity from components of the pathogen that can have deleterious effects on the recipient of the vaccine (Mills, 2001). Concern over potential side effects has driven the development of safer, but sometimes less immunogenic, sub-unit vaccines. Indeed, use of whole cell vaccines against pertussis was phased out in the 1990's following concerns about reactogenicity and potential toxic effects. These were replaced with acellular vaccines which are composed of antigenic components of the organism that are purified free from toxic contaminants or made in a recombinant form and may be modified to retain immunogenicity yet lack toxicity (Marciani, 2003). Acellular pertussis vaccines composed of detoxified PT, with a combination of FHA and agglutinogens were successful at inducing immunity but were of a lesser potency than the whole cell vaccines (Cherry, 2003). The development of techniques for large-scale recombinant protein preparations has facilitated the economical manufacture of safer sub-unit vaccines. This is discussed further in section 8.1.

1.3 The immune response

The immune response provides protection against infection and is essential to the hosts defence against invading microorganisims. The immune system may be broadly divided into innate and adaptive arms, each with its own distinct characteristics. Each arm of the immune response does not exist independently of the other and there is clear evidence that the innate immune response can direct the induction of adaptive immunity. The innate immune response is the first line of defence against invading organisms and includes physical barriers to pathogen infiltration and a

variety of phagocytic cells and molecules that rapidly deal with invading pathogens. The adaptive or acquired immune response is responsible for specific and prolonged immunity to pathogens. The adaptive response is responsible for the establishment of immunological memory, which allows for a rapid resurrection of the pathogen-specific response in the event of subsequent challenge by the same organism. The acquired immune response is mediated by T and B lymphocytes, whose activation is dependent upon innate immune cells. These T and B lymphocytes are specific for antigens from the pathogen and are known as effector cells. Some of these cells develop into T and B memory cells, that can quickly proliferate once they re-encounter a specific pathogen-derived antigen during a subsequent infection and activate a rapid effective immune response to clear the invading pathogen. The induction of a potent memory T and B cell response is the basis of successful vaccination.

1.3.1 Innate immunity

The innate immune response is the first line of defence against invading pathogens and also educates and initiates the acquired immune response against the pathogen (Hornef *et al.*, 2002). The innate defences consist firstly of physical barriers to pathogens, including the skin and mucosae and also the cilia of the trachea that function to maintain a clear respiratory system. The cells of the mucosa form a tight barrier that prevents the entry of large macromolecules, such as large proteins, viruses, parasites and bacteria, into sensitive somatic sites. However, this tight barrier does allow the diffusion of water and the passage of ions. As well as the physical barrier created by the epithelial cells, a layer of glycoproteins forms a barrier that prevents direct contact of pathogens with the epithelium. A layer of mucus is also secreted by goblet cells along the respiratory tract and this functions in a similar fashion, preventing direct contact of pathogens with cells and facilitates their expulsion by the cilia that line the airways (Cario *et al.*, 2002).

However, many pathogens have evolved ways to breach the physical barriers of the epithelium. This assault results in the activation of complement and the mobilisation of innate effector cells to the site of infection to begin in the elimination of the invading organism. The complement system is made up of a large number of

plasma proteins that react with one another to opsonise pathogens, marking them for destruction by phagocytic cells. Other components of the complement system, once activated, can form a membrane attack complex that can directly eliminate pathogens and infected cells. The innate response includes phagocytic cells, which include macrophages, dendritic cells (DC) and polymorphonuclear cells that have direct effector function in the clearance of pathogens and in the initiation of an adaptive immune response. These cells constantly sample their environment for danger signals secreted by or expressed on the surface of pathogens and may themselves engulf and destroy pathogens. By secreting a large variety of soluble signalling proteins such as cytokines and chemokines, activated innate cells can regulate the function of distant innate phagocytic cells and signal for the infiltration of more innate cells to assist in clearance of the infection (Cassatella, 1995, Melchjorsen *et al.*, 2003). These soluble protein signals are also important in the regulation and activation of the adaptive immune response against the pathogen. In addition to random sampling of their external milieu, these innate phagocytic immune cells also express receptors for the recognition of conserved pathogen associated molecular patterns (PAMPs) (Banchereau and Steinman, 1998, Medzhitov and Janeway, 1997).

1.3.2 Pattern recognition receptors

Cellular recognition of pathogens is mediated by a set of conserved receptors that are collectively referred to as pattern recognition receptors (PRRs). These receptors recognise conserved pathogen-associated molecular patterns that are shared by a large group of microorganisms and are invariant among entire classes of pathogens (Janeway and Medzhitov, 1998). These receptors, which include mannose binding protein, Toll-like receptors (TLRs), integrins and complement receptors, each bind a specific motif shared by products or components of a variety of pathogens. The resulting intracellular signalling cascades cause the innate cell to initiate an immune response against the invading microorganism (Gordon, 2002, Medzhitov and Janeway, 1997).

1.3.3 Toll-like receptors (TLRs)

The TLRs are an important group of PRRs that are a key component of the innate immune system. TLRs detect microbial infection and activate antimicrobial host defences (Iwasaki and Medzhitov, 2004). Although the exact number of genes differ between species, there appear to be 10 to 15 mammalian TLRs (Akira, 2003, Iwasaki and Medzhitov, 2004) that recognise gram-positive and gram-negative bacteria, fungi and viruses and activate multiple steps in inflammatory reactions that help to eliminate invading pathogens.

TLR4 was the first mammalian TLR to be identified, and it was shown to be necessary for the induction of an inflammatory immune response against lipopolysaccharide (LPS) (Hoshino *et al.*, 1999), a major structural component of Gram-negative bacteria. The necessity for a functional TLR4 was demonstrated in the C3H/HeJ strain of mice, which are insensitive to doses of LPS that are otherwise lethal in the wildtype, C3H/HeN strain. TLR4-defective mice are LPS hyporesponsive and exhibit an altered immune response when compared to the wildtype strain. Macrophages from C3H/HeJ mice fail to produce inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 in response to LPS stimulation. The gene responsible for LPS insensitivity was later identified in these mice and analysis of this gene in C3H/HeJ mice revealed a proline to histidine mutation at position 712 within the cytoplasmic signalling domain of TLR4 (Poltorak *et al.*, 1998, Qureshi *et al.*, 1999). This mutation results in ineffective LPS signalling through the TLR4 pathway in C3H/HeJ mice. The importance of TLR4 in LPS responsiveness was further confirmed by knocking out the *Tlr4* gene in mice. This knockout mutation completely inhibited LPS signalling (Hoshino *et al.*, 1999).

Since the identification of TLR4 and the discovery that it could mediate immune responses induced by a pathogen derived molecule, additional TLRs have been identified based on functional experiments and *in silico* sequence alignments. TLR1 has been shown to bind synthetic lipoproteins (Takeuchi *et al.*, 2002). TLR2 was shown to mediate immune responses induced by zymosan from yeast, peptidoglycan (Yoshimura *et al.*, 1999) and lipoteichoic acid from Gram-positive bacteria (Schwandner *et al.*, 1999). TLR3 functions in the recognition of double-stranded RNA from viruses and the

synthetic analogue, poly I:C (Alexopoulou *et al.*, 2001). TLR5 is involved in the identification of flagellin, a structural component from the flagella of bacteria (Hayashi *et al.*, 2001). Artificial compounds, such as imidazoquinoline, which is used in antiviral and antitumor therapy, activate TLR7 (Hemmi *et al.*, 2002). Single stranded viral RNA from different species is recognised by TLR7 and TLR8 (Diebold *et al.*, 2004, Heil *et al.*, 2004). TLR9 recognises CpG motifs in bacterial DNA (Hemmi *et al.*, 2000). It has also been reported that several TLRs can co-operate in the recognition of pathogen-derived molecules; TLR2 and TLR6 cooperate to recognise pathogen derived molecules such as lipoprotein from *Mycoplasma* that neither TLR can recognise alone (Ozinsky *et al.*, 2000, Takeuchi *et al.*, 2001). Co-stimulation of TLR3 or TLR4 together with stimulation of TLR8 has recently been shown to promote the secretion of IL-12 and IL-23 and the induction of Th1 cells (Napolitani *et al.*, 2005). Co-operative recognition allows this small set of receptors to respond to a wider variety of pathogen-derived molecules, which ultimately results in a more potent and rapid immune response against pathogens.

As well as being activated by molecules derived from pathogens, several endogenous molecules have been identified which can activate the immune system by signalling through the TLR pathways. It has been suggested that these endogenous TLR-ligands act as danger signals for the activation of the innate immune system and could be released by dying or stressed cells (Gallucci and Matzinger, 2001). Fibronectin, a molecule that is released from damaged tissue, has been shown to bind TLR4 (Okamura *et al.*, 2001). Heat shock protein (HSP) 60 has also been shown to activate the TLR4 pathway (Vabulas *et al.*, 2001). Cells produce HSP-60 during stress, which could be pathogen derived, and may signal the need for an innate response to be directed at the site of stress. Deficient clearance of apoptotic cells may allow host nucleic acids to become available to activate TLRs and inflammatory immune responses (Leadbetter *et al.*, 2002).

1.3.4 TLR signalling

While TLRs share many structural similarities, they differ in their expression patterns and the signal transduction pathways that are activated when they bind their

respective ligands. Indeed, the biological importance of multiple simultaneous activation of several TLRs by a pathogen is only beginning to be understood (Napolitani *et al.*, 2005). TLRs sense pathogens and activate antimicrobial and inflammatory responses and a subset of TLR-induced signals is devoted to the activation and control of the adaptive immune response against the pathogen.

TLRs are transmembrane proteins whose cytoplasmic domain has significant similarity to the receptor for members of the IL-1 family, which are potent signalling molecules that are secreted by immune cells. Both TLRs and IL-1 receptors have a conserved 200 amino acid motif called the Toll-IL-1 receptor-resistance (TIR) domain. Upon activation by binding of a ligand to a TLR, which may involve complexing of the ligand with further surface molecules such as LPS binding protein (LBP) and CD14 in the case of LPS, the cytoplasmic domain of the TLR recruits IL-1RI-associated kinase (IRAK)-1 via the adapter molecule MyD88 (Fig. 1.1). IRAK1 also associates with IRAK4, which is then activated and leads to the phosphorylation of IRAK1, that then associates with tumor necrosis factor receptor-associated factor 6 (TRAF6). This IRAK1-IRAK-4-TRAF6 complex initiates a kinase phosphorylation cascade that leads to the activation of the kinase IKK. Inactive IκB sequesters the transcription factor NF-κB in the cytosol of the cell. Upon activation, IKK phosphorylates IκB, leading to its degradation by the proteosome. This then frees NF-κB and allows it to translocate to the nucleus where it can bind promoter regions in DNA and activate the transcription of immune response genes (Akira, 2003, Akira and Hemmi, 2003).

The essential role for MyD88 in TLR and IL-1 signalling was demonstrated in knockout mice that were shown to be unresponsive to ligands for TLR4 (LPS), TLR2 (peptidoglycan), TLR9 (CpG DNA), TLR7 (imidazoquinolines) and to IL-1 stimulation (Akira and Hemmi, 2003). An additional adaptor molecule, TIR domain containing protein (TIRAP) or MyD88 adaptor-like (Mal), was identified and found to associate with the TIR domain of TLR4 but not with those of the other TLRs (Fitzgerald *et al.*, 2001). TIRAP/Mal is thought to associate with the MyD88-TLR4 complex and to be necessary for optimal signalling through the TLR4 receptor. In Mal or MyD88 knock out mice TLR4 signalling does occur, but at a reduced efficiency (Horng *et al.*, 2002, Yamamoto *et al.*, 2002). TIRAP/Mal has also recently been shown to be involved in the

TLR2 signalling pathway and mediates activation of NF-κB through TRAF-6 (Mansell *et al.*, 2004). Interestingly, a MyD88 independent signalling pathway has been identified for TLR4 signalling. MyD88 deficient macrophages were shown to retain responsiveness for LPS. This MyD88 independent pathway for LPS activation of innate cells has been shown to lead to the activation of interferon regulatory factor (IRF)-3 through the activation of the adaptor molecules TIR-domain containing adaptor-inducing interferon-β (TRIF) and TRIF related adaptor molecule (TRAM). IRF-3 is a transcription factor that translocates to the nucleus and activates genes, including those for type-I interferons that are involved in anti-viral responses (Kawai *et al.*, 2001b). A MyD88 independent pathway for IRF-3 activation was also shown to be activated following TLR3 activation and is dependant on activation of TRIF. It was initially thought that the signalling cascades downstream of each of the TLRs were identical. But it is now clear that despite the similarities in TLR signalling, significant differences exist and that individual TLRs activate different sets of response genes and that these are specific for the pathogen derived molecular patterns that each of the TLRs bind.

1.3.5 Dendritic cells (DC)

The innate and adaptive arms of the immune response function to eliminate pathogens and to generate protective adaptive immunity against infectious agents. Innate cells, such as macrophages, neutrophils and DC not only play an important role as phagocytic effector cells but also function as sentinels, constantly sampling for pathogens and pathogen-derived molecules, which they process and present to antigen-specific T cells (Banchereau and Steinman, 1998). DC are the most potent of the antigen presenting cells (APC) and play a critical role in the generation of an antigen-specific immune response by directing the differentiation of naïve $CD4^+$ T cells into functionally distinct T helper type (Th)1, Th2 or regulatory T (Tr) cell subtypes and the activation of cytotoxic T lymphocytes (CTL). DC are generated in the bone marrow and migrate as precursor cells to sites of potential entry of pathogens. Activation of immature DC (iDC) though binding of PRRs by pathogen-derived molecules is accompanied by maturation and homing to the local lymph nodes, where the mature DC present the pathogen derived antigenic peptides on surface expressed major

histocompatibility complex (MHC) molecules to naïve T cells. The secretion of regulatory cytokines and chemokines and the upregulation of costimulatory molecules on the DC direct the polarisation of the naïve T cell (Banchereau and Steinman, 1998, Iwasaki and Medzhitov, 2004, Kaisho and Akira, 2001). Each of these events are influenced by the PAMPs encountered by the DC. DC express many types of PRR, including C-type lectins, integrins, manose receptors and TLRs. TLRs and other PRRs are not universally expressed on all DC, and the family of PRRs expressed by a particular DC subset represent an important factor in the generation of pathogen specific effector responses.

Two major DC subsets, $CD11c^{+}$ myeloid DC and $CD11c^{lo}$ plasmatoid DC, have been described (Liu *et al.*, 2001). These DC subsets each express a differing complement of TLRs as well as having differences in cytokine expression. For example, myeloid DC express TLR4 but low levels of TLR9 and are consequently more responsive to LPS. Inversely plasmatoid DC express high levels of TLR9 and lower levels of TLR4 allowing their activation by CpG DNA, while TLR7 is expressed on both DC subtypes. Compared to myeloid DC, plasmacytoid DC also secrete higher levels of type I interferons, which have a role in antiviral immunity.

The ability to respond to LPS, CpG motifs, bacterial derived proteins and derivatives of yeast and viruses make DC extremely effective cells in immune surveillance and reflect their importance in detecting invading microorganisms and activating the acquired immune response. Immature DC are found primarily in the periphery, where they constantly sample exogenous proteins by endocytosis and other large molecules and pathogens by phagocytosis or macropinocytosis, which are then processed and presented to naïve T cells on MHC class-II molecules (Banchereau and Steinman, 1998). DC and other cells of the innate immune system can also be activated by pathogen derived molecules which do not necessarily bind PRRs but never the less have immune stimulatory ability. These include many bacterial toxins, secreted proteins from parasites and other enzymatically active proteins from pathogens that can bind to the cell surface and be internalized by these cells and stimulate intracellular signalling in the DC. Maturation and activation of the iDC to different levels of activation and cytokine synthesis occurs as the DC migrates to the local draining lymphoid organ. DC

activated by different pathogen-derived molecules express distinct patterns of co-stimulatory molecules and cell surface markers and secrete different cytokines and chemokines (McGuirk and Mills, 2002). iDC express receptors for inflammatory chemokines, such as CCR5 and CCR6 on their cell surface and are responsive to inflammatory mediators such as IL-1 and TNF-α, as well as to pathogen-derived molecules, such as LPS. During the process of DC maturation, inflammatory cytokine and chemokine receptors are down regulated and other chemokine receptors, such as CCR7 that binds MIP-3β are upregulated. The latter facilitates migration of DC from peripheral tissues to the afferent lymphoid areas (Melchjorsen *et al.*, 2003) and from there to the T cell areas of the draining lymph nodes, where they present antigen to naïve T cells (Sallusto and Lanzavecchia, 2000). The mature DC lose the ability to capture and process antigen but have enhanced antigen presentation capacity as a consequence of enhanced surface expression of MHC class-II and co-stimulatory molecules such as CD80, CD86 and CD40. CD80/86 expressed on DC binds its ligand CD28 on the T cell and provides the second signal for T cell activation. CD40 binding with CD40L, OX40 with OX40L, intercellular adhesion molecule (ICAM)-I with leukocyte function-associated antigen (LFA)-I facilitate T cell interaction with the APC and help trigger an antigen specific adaptive immune response (Banchereau and Steinman, 1998, Iwasaki and Medzhitov, 2004, Steinman, 1991).

DC can be divided into subtypes based on their PRR and surface marker expression pattern, anatomical location or specialisation to deal with certain types of invading pathogens. A classification based on the adaptive immune response elicited by the DC and the surface marker and cytokine profile of the DC has revealed three main subtypes, termed DC1, DC2 and DCr, which selectively induce specific subpopulation of $CD4^+$ T cells, termed T helper 1 (Th1), Th2 and T regulatory 1 (Tr1) respectively (Fig. 1.2) (Banchereau and Steinman, 1998, McGuirk and Mills, 2002). Certain pathogen derived molecules, including LPS, CpG, dsRNA and pertussis toxin (PT) activate DC1 that drive the differentiation of Th1 cells (de Jong *et al.*, 2002, Hemmi *et al.*, 2000). In contrast, products of helminth parasites (Whelan *et al.*, 2000), CT (Gagliardi *et al.*, 2000), and yeast hyphae (d'Ostiani *et al.*, 2000) promote the activation of DC2 that drive the differentiation of Th2 cells. Finally, filamentous

haemagglutinin (FHA) from *Bordetella pertussis* (McGuirk *et al.*, 2002) and CT (Lavelle *et al.*, 2003) activate DCr that promote the differentiation of IL-10- secreting type 1 Tr (Tr1) cells. Regulatory cytokines secreted by cells of the innate immune system in response to pathogen-derived molecules also play a critical role in the differentiation of naïve $CD4^+$ T cells. IL-12, IL-23 and IL-27 (Hochrein *et al.*, 2001, Trinchieri, 2003) and type-1 interferons (Kadowaki *et al.*, 2000) promote the differentiation of Th1 cells, while IL-4, IL-6 and IL-10 have all been shown to play a role in activation of Th2 cells (Rincon *et al.*, 1997). IL-23 promotes a subpopulations of IL-17 producing T cells (Hunter, 2005). Finally IL-10, as well as IL-4, has been implicated in promoting the induction of Tr1 cells (McGuirk *et al.*, 2002, Mendel and Shevach, 2002, Moser and Murphy, 2000).

1.3.6 Macrophages

Macrophages are the scavenger cells of the innate immune system and like DC are derived from the large pool of circulating monocyte precursors that arise in the bone marrow and demonstrate a developmental plasticity until the late stages in differentiation (Chomarat *et al.*, 2000) where they come to reside in the tissues, becoming differentiated into mature resident macrophages following 1 to 3 days circulating in the blood. However, under inflammatory conditions circulating macrophage precursors are rapidly recruited to sites of injury and pathogen invasion (Taylor and Gordon, 2003). Macrophages play an important role in the elimination of apoptotic cells, cells damaged by assault or injury or cells infected with pathogens.

When activated, macrophages have a reduced ability to proliferate. Macrophages, like DC, express a wide variety of PRRs, including integrins, TLRs and C-type lectins, that allow the macrophage to monitor its environment and to become activated upon interaction with a pathogen derived molecule. In addition, macrophages respond to the external cytokine and chemokine environment through the expression of the appropriate receptors, allowing macrophages to respond to other immune cells and to migrate to the site of inflammation. When activated the expression of MHC class-II molecules is upregulated on macrophages and consequently their antigen presentation abilities are enhanced. Macrophages also secrete cytokines and chemokines in response

to the immune stimulus that they encounter, thereby signalling for the activation of other immune cells. While macrophages are unable to activate naïve T cells to differentiate into effector T helper cells, they can present antigens to effector T cells and influence the polarisation of naïve T cell and DC maturation. Macrophages also take up pathogens through opsonization via Fc receptors following binding of antibodies and complement, thus facilitating phagocytosis and elimination of the pathogen. (Taylor and Gordon, 2003, Taylor *et al.*, 2005).

1.3.7 Acquired immunity

Acquired immunity is characterised by both specificity and memory and is mediated by both T and B lymphocytes (T and B cells). T and B cells express receptors on their surface that are specific for a single antigenic epitope. The extensive repertoire of receptors generated results from genetic recombination during the development of T and B cells. T cells are produced in the bone marrow, but mature in the thymus. B cells are produced in the bone marrow and also mature there. Effector T cells activate B cells, which differentiate into fully mature plasma cells that secrete antibodies specific for the antigen that aid in the clearance of the pathogen from the body.

1.3.8 T Lymphocytes

Naïve T cells require several signals to enter a fully mature and activated state. Binding of the T cell receptor (TCR) to antigen that has been processed into peptides and presented by MHC class II molecules on the surface of APC provides the first signal for T cell activation (Linsley and Ledbetter, 1993). A second signal is provided by CD80/86 (B7.1/B7.2) co-stimulatory molecules expressed on the surface of APC interacting with CD28 on the surface of the T cell (Kapsenberg, 2003, Sperling and Bluestone, 1996). Both TCR activation and co-stimulatory molecule interaction are necessary for the clonal expansion and activation of the effector functions of the T cell (Moser and Murphy, 2000, Robinson and O'Garra, 2002). Additional interactions between surface molecules of the APC and naïve T cell, such as CD40-CD40L binding,

are required for the activation of T cells and are critical in the induction of polarized adaptive immune responses (Schoenberger *et al.*, 1998). Stimulation of the TCR in concert with adequate co-stimulation induces the production of IL-2 from the $CD4^+$ cell. This cytokine promotes proliferation of T cells and is needed for the initiation of a variety of effector functions (Boise *et al.*, 1995). In addition, the subtype of T cell induced depends strongly on a third signal provided by cytokines and chemokines produced by cells of the innate immune system. The profile of secreted cytokines and expressed surface co-stimulatory molecules expressed by DC is influenced by the stimulus from pathogens and the conditions under which the DC are primed (Kalinski *et al.*, 1999, Kapsenberg, 2003).

1.3.9 Cell mediated immunity

$CD4^+$ T helper (Th) cells play a vital role in protection against pathogens that cause infectious diseases. $CD4^+$ T cells recognise exogenous antigen presented by APC in the context of MHC class-II molecules on their cell surface. Somatic gene rearrangements within the TCR cluster of genes during the differentiation of these cells generates a population of cells with a diverse array of T cell receptors, which allow the recognition of a vast selection of antigenic peptides (Rajewsky, 1996). Naïve T cells circulate through the blood and lymph until they encounter an APC bearing antigen bound to MHC class-II and expressing the correct array of co-stimulatory molecules on its surface. The interaction of the TCR with the antigen-MHC complex determines the antigen specificity of the response. This interaction usually occurs in draining lymph nodes but also in the spleen or peripheral lymph nodes, to which the APC may have migrated and where the clonal expansion of the activated T cell can take place (Banchereau and Steinman, 1998, Iwasaki and Medzhitov, 2004, Moser and Murphy, 2000). The initiation of protective adaptive immunity depends upon the naïve T cell receiving appropriate stimulation with antigen, co-stimulatory molecules and cytokines from the DC.

1.3.10 T cell differentiation

Naïve $CD4^+$ T cells were first reported to differentiate into two distinctly polarized subtypes. Type 1 and type 2 helper (Th1/Th2) subpopulations, identified on the basis on their cytokine profiles and function (Mosmann *et al.*, 1986). Th1 cells secrete pro-inflammatory cytokines, primarily interferon (IFN)-γ and also induce cell-mediated immunity such as macrophage activation and the induction of pro-inflammatory cytokines from innate cells. Th1 cells also function in the induction of antigen specific immunoglobulins, in particular the immunoglobulin (Ig) G2a subclass of IgG, which are involved in complement fixation, opsonization and clearance of bacteria (Mahon *et al.*, 1995). The Th2 pathway of differentiation is thought to be the default pathway of development for naïve $CD4^+$ T cells (Jankovic *et al.*, 2002). In the absence of a potent stimulus from the antigen or in the context of proteins that lack immunomodulatory activity, Th2 cells appear to be induced. However, the activation of Th2 cells by pathogen derived molecules has also been demonstrated (Stavnezer, 1996). A Th2 response induced in the host would be beneficial for the survival of pathogens, such as *Bordetella pertussis* and *Mycobacterium tuberculosis*, whose clearance by the immune system is facilitated primarily by Th1 cells (Mills, 2001, O'Garra and Murphy, 1994). Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, and also play a role in the induction of antigen specific IgG1 subclass of antibodies that neutralise toxins and aid in the recruitment of other cells and molecules that help to eliminate the pathogen.

In addition to Th1 and Th2 cells, additional subtypes of $CD4^+$ T cells have since been described. However, a unique cytokine profile, rather than the expression of cell-surface markers, has been used to define these populations (Mills, 2004). Th0 cells secrete a mixture of both Th1 and Th2 cytokines in varying levels and are thought to be an intermediate precursor population (Chen *et al.*, 1994, Elson *et al.*, 1995). More recently a number of T cell subtypes with regulatory activity have been identified. One such population, termed type 1 regulatory T cells (Tr1) produces high concentrations of IL-10 and little or no IFN-γ or IL-4 while IL-5 may be produced, but in low amounts. (Doetze *et al.*, 2000, Higgins *et al.*, 2003, Lavelle *et al.*, 2003, Marshall *et al.*, 2003, McGuirk *et al.*, 2002, McGuirk and Mills, 2002, Satoguina *et al.*, 2002). It was initially reported that Tr1 cells could be generated *in vitro* by repeated antigen stimulation in the

presence of IL-10 (Groux *et al.*, 1997). It was later demonstrated that interaction of certain pathogen derived molecules that enhanced DC IL-10 production and partially matured the DC promoted the induction of Tr1 cells *in vivo* (McGuirk *et al.*, 2002). Tr1 cells have been shown to suppress inflammatory cytokine production by Th1 cells (Lavelle *et al.*, 2003, McGuirk *et al.*, 2002), through the action of the secreted IL-10 (Doetze *et al.*, 2000, MacDonald *et al.*, 2002, Satoguina *et al.*, 2002). These Tr1 cells may also prevent the activation of naïve T cells into Th1 cells by inhibiting IL-12 production and downregulating the expression of co-stimulatory markers, especially CD40 (Lavelle *et al.*, 2003, Martin *et al.*, 2003). An additional regulatory T cell population, known as Th3 cells, have been identified. Th3 cells secrete high levels of the anti-inflammatory cytokine TGF-β, which have protective properties against inflammatory and autoimmune diseases, such as multiple sclerosis (Chen *et al.*, 1994).

The development of a fully active and appropriately polarized immune response is required for the induction of protective and long lasting immunity against pathogens (Spellberg and Edwards, 2001). Th1 cells are important in protective immunity against intracellular pathogens. The development of a Th2 response can exacerbate infection or result in harmful pathology with certain pathogens, including *Leishmania* and respiratory syncytial virus (RSV) respectively (Hussell *et al.*, 1997, Miralles *et al.*, 1994). In contrast, Th2 cells are important in protection against extracellular pathogens, including yeasts and helminth parasites. The control of inflammatory immune mediated diseases such as Crohn's disease is facilitated by the induction of a Th2 or Tr cells (Groux *et al.*, 1997). Many factors have been shown to influence the development of polarized T cell responses. Antigen dose and the route of antigen delivery have been implicated in the regulation of T cell differentiation (Conway *et al.*, 2001, Ruedl *et al.*, 2000). The expression of certain panels of co-stimulatory molecules have also been shown to influence the development of each of the T cell subpopulations. OX40 ligand expressed on the surface of DC binds to OX40 on the surface of the T cell and promotes proliferation and the induction of cytokine secretion from the T cell. The ligation of OX40 to OX40-Ligand on the surface of the T cell has been reported to be a factor that influences the development of Th2 type responses, through the induction of increased levels of IL-4 (Flynn *et al.*, 1998, Kapsenberg, 2003). However, ligation of

ICAM-1 on the surface of the DC to its receptor (LFA-1) on the T cell surface suppresses Th2 cell development, while promoting the polarisation of Th1 cells (Kapsenberg, 2003, Murphy and Reiner, 2002). CD40 interaction with CD40L promotes IL-12 production and polarisation of a Th1 subtype, whereas absence of CD40 facilitates the induction of Tr1 cells (Lavelle *et al.*, 2003, Martin *et al.*, 2003).

1.3.11 Influence of cytokines on T cell differentiation

The cytokines present in the external milieu during T cell differentiation have been shown to influence the development of naïve $CD4^+$ T cell into one of the polarised subtypes (de Jong *et al.*, 2002, Moser and Murphy, 2000, Rincon *et al.*, 1997, Spellberg and Edwards, 2001). IL-12, and IL-27 have been shown to be important in the development of Th1 cells from precursors (Mills, 2004). It has also been suggested that a sustained level of IL-12 is important in the maintenance of Th1 responses. However, it has been recently shown that IL-23 also promotes a distinct subtype of IL-17 secreting T cells (Hunter, 2005). IL-4 promotes the differentiation of Th2 cells and also inhibits the differentiation of Th1 cells, even in the presence of IL-12 (O'Garra and Arai, 2000). The exact source of IL-4 required for Th2 induction has not been fully defined, although it has been reported that naive T cells produce endogenous IL-4 and thereby drive their own differentiation towards the Th2 pathway (Noben-Trauth *et al.*, 2000). This may add strength to the hypothesis that Th2 is the default pathway of differentiation of $CD4^+$ T cells. Although capable of promoting Th2 development, IL-4 is not essential. Mice deficient in IL-4 signalling can still develop potent Th2 responses (Brewer *et al.*, 1999). IL-18 is also important in the differentiation of T cells and plays an important role in combination with IL-12 in the induction of Th1 cells (Robinson and O'Garra, 2002). However, it has also been shown that IL-18 either alone or with IL-4 has been shown to induce Th2 cells (Xu *et al.*, 2000). IL-13 has been shown to promote the development of Th2 responses during *Leishmania major* infection (Matthews *et al.*, 2000). IL-10 appears to be the most important cytokine in the development of Tr1 cells (McGuirk and Mills, 2002); the induction of IL-10 and suppression of IL-12 from DC enable them to drive the differentiation of Tr1 cells (McGuirk *et al.*, 2002). It is clear that many cytokines are necessary to drive polarized

T cell responses. But it is the spectrum of cytokine in the local environment as well as the direct stimulation provided by the APC which dictates the developmental path of the naïve T cell.

1.3.12 Memory T Cells

Following the clearance of the infection, and removal of the initial signal that provoked the immune response, the effector T cells die by apoptosis or programmed cell death. However memory T cells will also have developed over the course of the infection, each with the same antigen specificity of the effector T cell (Farber, 2003). These long-lived memory T cells are distinct from naïve T cells and are closer to effector T cells in terms of surface marker expression. In the event of subsequent infection these memory T cells undergo rapid clonal expansion and activation upon exposure to antigen and co-stimulatory molecules on the APC and then reactivate the acquired immune response. The goal of a successful vaccine is to generate a high frequency of memory T cells specific for the pathogen, that will be activated following infection and mediate rapid clearance of the pathogen, with minimum pathology.

1.3.13 B cells

Once immature B cells encounter antigen, these lymphocytes mature to plasma cells, which then proliferate and begin to express soluble antibodies specific for the antigen (Bishop *et al.*, 2003). This antigen-specific antibody production may be elicited by the direct binding of antigen to antibody or Ig that is bound to the membrane of the immature B lymphocyte, which functions as a B cell receptor (BCR). Most antigens however require the interaction of a T helper (Th) cell with the B cell to stimulate antibody production. B cells are also APC and capture antigen via the BCR, and this is then internalized and processed before presentation on MHC class-II molecules on the cell surface. Activated $CD4^+$ Th cells, either Th1 or Th2 cells, which are specific for the antigen-MHC-II complex, bind the B cell via the TCR and through the interaction of surface markers, such as CD40 ligand and CD28, with their ligands, CD40 and CD80, on the surface of the B cell, and through the secretion of cytokines, activate B

cells to proliferate and develop into antibody-producing plasma cells (Clark and Ledbetter, 1994).

B cells can secrete five antibody isotypes, which may switch during the course of the maturation of the plasma cell (Manz *et al.*, 2005). IgM is the first Ig class produced by B cells and being a large pentamer does not diffuse well and is found primarily in the blood, where it functions to agglutinate antigen and activate complement, a system of soluble factors that mediate pathogen destruction. IgA is primarily found at mucosal sites and plays a role in preventing bacterial and viral attachment to host cells. IgE antibodies play a role in allergic responses and activate mast cells and basophils to release substances, such as histamine that can be involved in allergic responses. IgD has been identified and is primarily found on the surface of B cells and may be able to substitute for some of the functions of IgM. IgG is the most abundant isotype of immunoglobulins and to date four isotypes have been identified. They have a wide variety of functions including the activation of complement and the opsonization of antigen. The cytokines that are secreted by the T cells during the activation of the B cell are important in the antibody class and isotype secreted by the B cell. Murine IgG2a is induced by IFN-γ-secreting Th1 cells, which also inhibits the production of IgG1 and IgE. Inversely, IL-4, which is secreted by Th2 cells, promotes the production of IgG1 and suppresses IgG2a and also IgM production (Coffman *et al.*, 1993, Lutz *et al.*, 1998, Purkerson and Isakson, 1992). Analogous to the induction of memory T cells, after the infection has been cleared and the antigenic stimulus has been removed, the activated B cells die by apoptosis leaving only memory B cells that are long lived and persist retaining the ability to rapidly activate and proliferate in response to a subsequent encounter with the antigen.

1.4 Modulation of the immune response

Inflammation and the immune response to pathogens is regulated by several host regulatory mechanisms, including the release of anti-inflammatory cytokines by cells of the innate immune systems in response to conserved PAMPs (Redpath *et al.*, 2001, Reed, 1999). In addition, the adaptive immune system plays a role in modulating the

control of infection through the induction of pathogen-specific regulatory T cells. (Mills, 2004). Pathogens have evolved strategies to subvert immunes responses in the host. However, it is also possible that pathogens could exploit the host immune system by promoting the induction of anti-inflammatory and regulatory responses to subvert the inflammatory response required to clear the infection.

1.4.1 Adjuvants

Adjuvants are substances that when administered in combination with a specific antigen produce a more robust immune response than administration of the antigen alone. Substances such as oil and lecithin have been shown to increase the adjuvanicity of bacterial toxoids used as vaccines against tetanus and diphtheria (Gupta and Siber, 1995). Many molecules have been evaluated as potential adjuvants for vaccines and several have demonstrated the ability to improve the potency of the antigen-specific response but few have been free of adverse reactions (Marciani, 2003). The use of sub-unit vaccines has now become the desirable method of inducing immunity to infectious diseases in humans. Sub-unit vaccines are composed of antigenic components of pathogens, and have the advantage of being free of toxic components but these preparations usually have low immunogenicity when compared to whole cell vaccines, which are whole pathogens that have been killed or attenuated. One reason for this may be that the danger signals of the pathogen, including TLR agonists, have been removed from the sub-unit preparation in order to reduce toxicity; these molecules have potent immunomodulatory activity and function as adjuvants in whole cell vaccines. One solution to the reduced potency of sub-unit vaccines is to increase the immunogenicity of the sub-unit vaccine preparation by the addition of a non-toxic molecule that acts as an adjuvant.

Currently the only adjuvants licensed for human use are aluminium salts (alum), an emulsion of oil and surfactants, called MF-59 (Gupta *et al.*, 1995) and immunopotentiating reconstituted influenza virosomes (IRIV) (Pashine *et al.*, 2005). While not effective at inducing the required immune response against all pathogens, alum has been used successfully in many human vaccines. However, alum principally

enhances humoral immunity and promotes the induction of Th2 cells making it less suitable for use in vaccines against intracellular pathogens and viruses (Brewer *et al.*, 1996, Sun *et al.*, 2003). The use of well defined adjuvants that bias an immune response towards an inflammatory, anti-inflammatory or regulatory phenotype as well as augmenting the strength of the immune response, will greatly assist in is the design of successful subunit vaccines.

1.4.2 Immunomodulatory molecules

Pathogens have evolved many mechanisms to regulate the immune system and persist in the host. These strategies include the production of molecules that interfere with cellular functions, including antigen processing and the subversion of phagocytosis and killing of innate cells (Mills, 2004, Mills and Boyd, 2005). Pathogen-derived molecules that activate innate and consequently adaptive immune responses have considerable potential for use in the development of the next generation of adjuvants. In particular the identification of molecules that polarise T cell responses to a distinct subtype have great promise for use as adjuvants. However, the immunomodulatory capacity of any such molecule would need to be fully characterised and its safety demonstrated.

1.4.2.1 CpG Motifs of Bacterial DNA

Unmethylated CpG motifs, which are generally absent from or present at lower frequencies in mammalian DNA, are potent adjuvants. CpG motifs bind to TLR9 on innate immune cells and stimulate a potent immune response that includes B cell proliferation and activation of innate cells (Hemmi *et al.*, 2000). Interestingly, similar methylated sequences do not appear to be immunostimulatory. The potential of CpG as an adjuvant and immunomodulator has been investigated using synthetic oligodeoxynucleotides (CpG-ODN), which have the 5'-purine-purine-CpG-pyrimidine-pyrimidine-3' motif. CpG-ODN has been shown to direct the activation of monocytes, DC and macrophages and to trigger the secretion of inflammatory cytokines, such as

TNF-α, IL-6 and IL-12 (Krieg, 2002). CpG-ODN have also been shown to direct the maturation of DC characterised by the upregulation of surface molecules such as MHC class-II, CD80 and CD86 (Askew *et al.*, 2000, Krug *et al.*, 2001). *In vivo*, CpG-ODN motifs have been shown to induce cells that produce IL-12, TNF-α and IFN-γ with little or no production of antigen-specific Th2 type cytokines (Lore *et al.*, 2003) and has also been shown to act as an adjuvant for malaria (Su *et al.*, 2003) and *Leishmania major* (Shah *et al.*, 2003) vaccines and is a potent inducer of mucosal immunity (Harandi and Holmgren, 2004). From the profile of innate and adaptive cytokines induced by CpG-ODN, it is considered to be a Th1 polarising immunomodulator.

1.4.2.2 Cholera Toxin

Cholera toxin is a member of the AB class of bacterial toxins and is composed of two subunits. The 55kDa pentameric B subunit forms a cylinder-like structure (Sixma *et al.*, 1993) and is responsible for binding of the toxin to the target cell via the GM1 ganglioside (Holmgren *et al.*, 1973). The A subunit contains the catalytic domain of the protein and has ADP-ribosylating activity that activates the GS_{α} GTP-binding protein, which in turn permanently activates the host adenylate cyclase in eukaryotic target cells (Gill and Woolkalis, 1991, Holmgren, 1981). This converts ATP to cyclic adenosine monophosphate (cAMP) and results in the accumulation of supra physiological level of this intracellular signalling molecule. This cAMP accumulation activates protein kinase A (PKA) that directs many physiological and immunological functions (Hofmann *et al.*, 1993). CT has been shown to act as an adjuvant in several animal models (Lycke, 1997, Rappuoli *et al.*, 1999, Williams *et al.*, 1999), although it is too toxic for clinical use in humans (Pizza *et al.*, 2001). Site-directed mutagenesis has been employed to generate recombinant derivatives of CT that are less toxic or have reduced enzymatic activity, but which retain adjuvant and immunomodulatory activity (Baudner *et al.*, 2003, Douce *et al.*, 1997, Giuliani *et al.*, 1998, Pizza *et al.*, 2001, Yamamoto *et al.*, 1997). CT has been shown to modulate the adaptive immune response enhancing the generation of Th2 (Marinaro *et al.*, 1995, Xu-Amano *et al.*, 1994) and Tr1 type cells *in vivo* (Lavelle *et al.*, 2003).

CT has also been shown to activate innate cells, modulating the surface expression of co-stimulatory molecules, including upregulating CD80 and CD86 and downregulating CD40 and ICAM-I expression. CT has also been shown to modulate cytokine secretion by DC and macrophages in response to TLR agonists, such as LPS and CpG, suppressing IL-12p70 and Il-12p40 and synergizing with LPS to upregulate IL-10 production (Lavelle *et al.*, 2003). The sustained increase in the levels of intracellular cAMP have been identified as one of the main mediators of the action of CT (Bagley *et al.*, 2002a). Synthetic pharmacological agents that increase cAMP levels, such as d-cAMP and forskolin, have also been shown mimic the effects of CT. An inhibitor that blocks PKA activation was also shown to abrogate the effects of CT and the cAMP elevating agents. However, the pentameric B subunit of CT, which lacks the catalytic domain, does modulate immunity, enhancing responses to a co-administered antigen but inhibiting inflammatory responses (Coccia *et al.*, 2005, Lycke, 2005). This demonstrate that CT and synthetic molecules that upregulate cAMP are potent immunomodulators.

1.4.3 Mucosal vaccines and adjuvants

Mucosal vaccines are easier to deliver compared to those delivered by injection, which helps to improve patient compliance, as well as eliminating problems with disposal of contaminated needles. Mucosal delivery may also confer protection at the site of infection by inducing local as well as systemic immunity. However, the generation of a sufficiently strong immune response by mucosal vaccination with purified or recombinant protein antigens requires the use of an adjuvant in the vaccine formulation (O'Hagan and Valiante, 2003). While alum is the most widely used vaccine adjuvant it is not used for mucosally delivery vaccines. However, the use of pathogen and plant derived molecules with mucosal adjuvant activity have shown great potential in their ability to stimulate both systemic and local immunity activity (Giuliani *et al.*, 1998, Lavelle *et al.*, 2001). Additionally the induction of antibodies with bactericidal activity by mucosal vaccines is also important in their efficacy (Bowe *et al.*, 2004).

Mucosal vaccines with appropriate adjuvants have the potential advantage of preventing the initial interaction between invading pathogens and the cells of the

mucosa and thereby aiding in the elimination of the pathogen before disease is established (Kiyono and Fukuyama, 2004). Understanding the mechanism of action of pathogen-derived products as mucosal adjuvants in mice opens up the possibility of developing safe and efficacious mucosal vaccines for use in man. A wide variety of ligands of TLRs and other PRRs have shown potential as novel mucosal vaccine adjuvants. LPS is a potent adjuvant and is used widely experimentally, but is too toxic for clinical use (Piggott *et al.*, 2005). Monophosphoryl lipid A, a derivative of LPS lipid A from *Salmonella enterica* like LPS binds to and activates signalling through TLR-4, but is less toxic and is a promising adjuvant for the generation of both mucosal and systemic immune responses (Yang *et al.*, 2002). Synthetic oligodeoxynucleotides containing unmethylated CpG motifs are agonists for TLR9 and promote DC activation and Th1 type responses and have been shown to be a strong adjuvant for vaccines delivered by the mucosal route (Harandi and Holmgren, 2004). In addition to TLR ligands, other pathogen-derived molecules have shown promising potential as mucosal adjuvants. Both FHA and PT from *B. pertussis* have been shown to have adjuvant activities and modulate immune responses at mucosal surfaces (Cropley *et al.*, 1995, Poulain-Godefroy *et al.*, 2003).

Cholera toxin (CT) from *Vibrio cholerae* and heat labile enterotoxin (LT) from enterotoxigenic strains of *E. coli* are potent mucosal adjuvants but can have adverse effects *in vivo* (Williams *et al.*, 1999). Both molecules are composed of an enzymatically active A subunit with adenosine-diphosphate (ADP)-riboslytransferase activity and a pentameric B oligomers that binds GM1-ganglioside on the surface of cells. Co-administeration of CT or LT with antigen by the oral or nasal route induces potent antigen-specific mucosal and systemic antibody responses (Takahashi *et al.*, 1996). CT also promotes a Th2-type response against the co-administered antigens (Lavelle *et al.*, 2003), while LT promotes the generation of Th1 and Th2 type cells (Bowe *et al.*, 2004). However, there are some safety concerns regarding the use of certain pathogen-derived molecules as nasal adjuvants. Radio-labeled CT has been found to traffic to the nervous system after intranasal immunisation (van Ginkel *et al.*, 2000). Similarly, radiolabeled LT has been detected in the olfactory bulb in BALB/c mice (Zurbriggen *et al.*, 2003), and has been implicated in the development of facial

paralysis in human recipients of a nasal influenza vaccine administered with LT as adjuvant (Mutsch *et al.*, 2004). CT and LT have also been shown to cause overt inflammation of the meninges of the olfactory bulb and severe lesions in intranasally immunised BALB/c mice (Bourguignon *et al.*, 1999). However, derivatives of LT have been engineered that have reduced or deleted enzymatic activity, yet retain certain of their immunomodulatory activities (Pizza *et al.*, 2001). Two such derivatives, LTR72, which has reduced enzymatic activity (Giuliani *et al.*, 1998), and LTK63, which lacks enzymatic activity (Baudner *et al.*, 2003), have been shown to retain the immunomodulatory ability of the wild type LT while lacking many of its deleterious effects. It is reported that clinical grade preparations of both derivatives fail to induce inflammation or cause histological changes in the olfactory bulb in mice or rabbits. The safety and efficacy of LTK63 has also been confirmed in a trial using it as an adjuvant for a nasally delivered influenza vaccine in human volunteers (Peppoloni *et al.*, 2003). However, concerns still remain over the safety of these derivatives as it has been reported that the B subunit of CT, which binds GM1-ganglioside, still traffics to the nervous system, where their effect is not clearly understood (Eriksson *et al.*, 2004).

1.4.3.1 Mucosal vaccine delivery systems

Although the use of adjuvants improves the immunogenicity of mucosally delivered vaccines, higher doses of antigen are required to ensure delivery of an adequate dose across the epithelium, this is especially a problem with orally delivered vaccines (Holmgren and Czerkinsky, 2005). Furthermore, the chemical, physical and enzymatic barriers present at the mucosa may significantly alter the vaccine as it crosses the epithelium (Kiyono and Fukuyama, 2004). The use of delivery systems can overcome some of these problems and can boost the immunogenicity of vaccines delivered mucosally (Lavelle, 2001). Encapsulation into micro-particles and coating of antigens with polymers have been shown to induce protective immunity against *B. pertussis*, influenza virus and simian immunodeficiency virus (SIV) with orally delivered immunogens (Vajdy and O'Hagan, 2001). Antigens can be incorporated within liposomes, which are made up of natural cell wall components such as phospholipids and cholesterol (Felnerova *et al.*, 2004). These structures allow them to

be easily absorbed onto cell membranes facilitating their uptake and the release of their contents. Intranasal immunisation with liposomes containing proteins of *N. meningitidis* has been shown to induce bactericidal responses (de Jonge *et al.*, 2004). Chitosan is a naturally derived polysaccharide and has been shown to promote transmucosal absorption of antigens and to enhance systemic and mucosal antibody responses to nasally delivered antigens (van der Lubben *et al.*, 2001). These delivery systems have the capacity to enhance the immunogenicity of antigens, especially when used with a powerful mucosal adjuvant.

1.5 *Bordetella pertussis*

B. pertussis is a Gram-negative bacterium and is the causative agent of whooping cough, a severe and prolonged infection in young children (Cherry *et al.*, 1988) and is also re-emerging as an important pathogen of adults in the developed world (Cherry, 2003). *B. pertussis* expresses many virulence factors including FHA, PT, tracheal cytotoxin (TCT), dermonecrotic toxin (DNT), LPS, fimbriae (FIM), pertactin (PTN) and adenylate cyclase toxin (CyaA). These have been shown to be essential for colonisation of the respiratory tract and to promote bacterial survival and pathogenesis (Mills, 2001, Smith *et al.*, 2001).

1.5.1 Adenlyate cyclase toxin

CyaA is encoded by the *cyaA* gene and is secreted into the external milieu by *B. pertussis* (Hewlett and Wolff, 1976), but can also be associated with the surface of the bacterium (Zaretzky *et al.*, 2002). CyaA is a monomeric 1706 amino acid polypeptide with bi-functional activity (Fig. 1.3), the N-terminal domain contains an enzymatic domain that converts adenosine triphosphate (ATP) to cAMP and the C-terminal domain is involved in membrane interaction and lysis of target cells (Ladant and Ullmann, 1999). The C-terminal 1306 amino acids contain a series of nonapeptide repeats involved in calcium binding (Rose *et al.*, 1995) similar to the RTX (repeat in toxin) family of exotoxins that have haemolytic and immune stimulatory ability (Coote,

1992). There are approximately 45 calcium-binding sites in the C-terminal domain of CyaA that can each bind a calcium ion. Binding of calcium ions by CyaA is essential to facilitate a protein conformation that allows the delivery of the N-terminal catalytic domain of CyaA into the cytosol of eukaryotic target cells (Rogel and Hanski, 1992, Rose *et al.*, 1995) and across artificial planar lipid bi-layers (Knapp *et al.*, 2003). Binding of calcium ions by CyaA also plays a role the activation of the catalytic domain by influencing the ability of CyaA to bind eukaryotic calmodulin (CaM) (Shen *et al.*, 2002), which is necessary for optimal enzymatic activity (Wolff *et al.*, 1980). The C-terminal domain of CyaA contains multiple hydrophobic domains that facilitate interaction of the protein with the membrane of the target eukaryotic cell and the formation of a pore (Benz *et al.*, 1994) through which potassium (Gray *et al.*, 1998) and sodium (Otero *et al.*, 1995) ions have been shown to escape.

1.5.2 CyaA catylases intracellular cAMP formation in target cells

Following binding to the eukaryotic target cell via the C-terminal domain, the CyaA protein undergoes a conformational change resulting in the N-terminal 400 amino acids being inserted through the plasma membrane directly into the cytosol of the target cell (Gray *et al.*, 2001, Ladant and Ullmann, 1999). This N-terminal region contains the enzymatic domain of the protein (Sakamoto *et al.*, 1992), which must bind eukaryotic calmodulin for optimal enzymatic activity of CyaA (Drum *et al.*, 2002, Gentile *et al.*, 1990, Rogel and Hanski, 1992, Wolff *et al.*, 1980). ATP is bound at the active site and is converted to cAMP, a powerful intracellular signalling molecule (Confer and Eaton, 1982). It has been shown that the N-terminal is cleaved from the CyaA protein and is released into the cytosol of the eukaryotic cell (Rogel and Hanski, 1992).

Many essential amino acid residues have been characterised and identified through functional studies and mutagenesis to be involved in the activity of CyaA. Histidine (His)^{63} and His^{106} are important residues in the catalytic site (Munier *et al.*, 1992). His^{63} when mutated to arginine (Arg), glutamic acid (Glu), glutamine (Gln) or valine (Val) was shown to result in the catalytic activity being reduced by 2-3 orders of magnitude. Substituting His^{106} with Arg, Gln or asparagine (Asn) decreased catalytic

efficiency by up to 90%. Lysine (Lys)[58] was shown to be important for ATP binding and when mutated reduced the catalytic activity of CyaA by 1000 fold (Ehrmann *et al.*, 1992). Lys[65] was also shown to be important in the binding of ATP, and its mutation to a non-reactive amino acid residue resulted in the enzymatic activity being reduced 500 fold (Glaser *et al.*, 1989). Serine (Ser)[66] has been shown to bind the γ-phosphate of ATP at the active site, which is important for the optimal activity of the enzyme (Drum *et al.*, 2002). The His[298] and tryptophan (Trp)[242] residues have been shown to be important for CaM binding (Glaser *et al.*, 1989). Substituting His[298] with a leucine (Leu) or Arg residue reduced the enzymatic activity by up to 90% but has been shown not to be directly involved in the catalytic step. Mutating Trp[242] resulted in the affinity of CyaA for CaM being reduced 1000 fold and without the binding of CaM the enzymatic activity is greatly reduced (Glaser *et al.*, 1989, Wolff *et al.*, 1980). Methionine (Met) residues at positions 109, 124, 145 are also critical for binding of CaM (Vougier *et al.*, 2004). These essential amino acid residues demonstrate that CyaA is a complex protein and that there are essential sites within the catalytic domain for ATP binding and utilization, as well as additional sites on the protein necessary for post-translational modification.

1.5.3 Post-translational acylation of CyaA

Post-translational acylation of CyaA by the specific accessory protein CyaC, is required for CyaA to lyse red blood cells (RBC) and T cells (Barry *et al.*, 1991, Hewlett *et al.*, 1993). CyaC is a cyclolysin-activating lysine acyltransferase with homology to the HlyC protein that specifically acylates the α-hemolysin (HlyA) of *E. coli* (Heveker *et al.*, 1994). Both *cyaA* and *cyaC* genes are encoded in the same operon on the *B. pertussis* chromosome and both can be transcribed simultaneously (Harvill *et al.*, 1999). This homology to HlyC led to the hypothesis that CyaC was post-translationally acylated with the transfer of a fatty acyl group from the acyl carrier protein (ACP) to the newly translated CyaA protein in *B. pertussis*. In a cell free *in vitro* system it was shown that the transfer of lauric acid (C12:0), myristic acid (C14:0) or palmitic acid (C16:0) to non-acylated CyaA could restore the lytic function (Heveker *et al.*, 1994).

Acylation of proteins can occur by palmitoylation in either of two forms, oxy- or thio-ester linkage to serine, threonine or cysteine residues or by myristoylation through amide linkage to the N-terminal glycine residues. However, CyaA activity was shown to be resistant to hydroxylamine treatment, indicating that the post-translational modification did not involve esterification (Hackett *et al.*, 1994). Hackett *et al.* (Hackett *et al.*, 1994) characterised CyaA purified from wildtype *B. pertussis* and a mutant strain lacking the *cyaC* gene. Using MALDI-TOF mass spectrophotometry, this study demonstrated that Lys^{983} was palmitoylated (the addition of a palmitoyl group) in wild type *B. pertussis*, while it was not detected in the mutant strain. The results also suggested that acylation may not be complete in the wildtype strain, with a small amount of unmodified protein present. This addition of a palmitoyl group was consistent with work by Issartel *et al.* (Issartel *et al.*, 1991) who showed that palmitoylation could occur through amide linkage of a lysine residue, but that there was not strong affinity for any particular fatty acid group. In this study they demonstrated that HlyA was palmitoylated at a lysine residue. Carrying on from this work, it was shown that acylation of Lys^{983} was 87% palmitoylation and 13% myristoylation of recombinant CyaA co-expressed with CyaC expressed in *E. coli* (Hackett *et al.*, 1995). This work also clearly demonstrated that recombinant CyaA was also acylated on Lys^{860} in a CyaC dependant manner, and that this acylation was exclusively palmitoylation, but that only 66% of the recombinant CyaA was acylated at Lys^{860}. However, CyaA purified from *B. pertussis* is exclusively palmitoylated at Lys^{983}, with less than 5% acylation of Lys^{860}.

It has also been demonstrated that acylation of Lys^{983} and Lys^{860} by CyaC are independent of each other (Basar *et al.*, 2001). Basar *et al.* also showed that Lys^{860} has an important role in the conformation CyaA takes to enable it to penetrate cell membranes. However, in this study, there was no evidence of myristoylation of either lysine residue and suggested that they were primarily palmitoylated. However they did report that an earlier batch of protein did have evidence of acylation, with palmitoleic fatty acid (C16:1). The authors concluded that subtle changes in growth and protein expression conditions in the bacterial culture may explain this difference. A more recent study demonstrated that recombinant CyaA expressed from *B. pertussis* but

encoded on a plasmid in the presence of the *cyaC* gene was palmitoylated at Lys^{983} and Lys^{860} and did not detect acylation with any other fatty acid (Havlicek *et al.*, 2001). Analysis of CyaA expressed in *E. coli* did, however, reveal a small amount of palmitoleic acid acylation.

It is possible that this difference between the fatty acid acylated to CyaA expressed in either *B. pertussis* or *E. coli* is related to the abundance of each fatty acid and the affinity of each fatty acid for ACP. While myristic acid has a high affinity for ACP it is present in relatively low amounts, while palmitic acid has a lesser affinity for ACP but is very common in bacterial systems (Issartel *et al.*, 1991). Palmitoleic acid displays moderate affinity for ACP and is relatively abundant in *E.coli.* While it is accepted that CyaC acylates CyaA at Lys^{983}, and also Lys^{860} in recombinant systems, the mechanism of acylation has not been investigated. However, a study of general fatty acid acylation of proteins and by drawing parallels to analogous work characterising the post translational modification of HlyA by HlyC of *E. coli,* a potential mechanism of acylation of CyaA by CyaC can be extrapolated (Fig. 1.4). In *E.coli,* the formation of halo-ACP is formed from free apo-ACP and CoA-4'phosphopopantenteine by halo-ACP-synthase in an ATP dependant manner (Polacco and Cronan, 1981). An acyl-ACP-fatty acid group is then formed from halo-ACP and free fatty acid by acyl-ACP-synthetase, again in an ATP dependant manner (Issartel *et al.*, 1991, Jackowski and Rock, 1986). This can then be utilised in membrane lipoprotein biosynthesis (Jackowski and Rock, 1986) or it comes together with non-acylated CyaA/HlyA and the specific accessory protein CyaC/HlyC, without the formation of any intermediate complexes (Issartel *et al.*, 1991). CyaA/HlyA receives the fatty acid group that is acylated through amide linkage, and which is resistant to hydroxylamine treatment (Issartel *et al.*, 1991). The mixing of free acyl-CoA, free fatty acid together with HlyA and HlyC does not result in the acylation of HlyA (Issartel *et al.*, 1991) demonstrating the necessity of the intermediate steps.

1.5.4 Adenylate cyclase toxin is required for virulence of *B. pertussis*

CyaA has been shown to be required for virulence of *B. pertussis* in a mouse model of infection (Goodwin and Weiss, 1990). Deletion of the gene dramatically

reduces the pathological effects of *B. pertussis* in infected mice, reducing numbers of bacteria in the lung (Gross *et al.*, 1992, Weiss *et al.*, 1984) and abolishing inflammatory cell recruitment (Harvill *et al.*, 1999, Khelef *et al.*, 1994). A protective role for CyaA in *B. pertussis* infection was indicated by the detection of high levels of anti-CyaA antibodies in the sera of *B. pertussis* infected patients (Farfel *et al.*, 1990) and mice (Betsou *et al.*, 1993). Specific monoclonal antibodies to CyaA enhance neutrophil phagocytosis of *B. pertussis in vitro* (Weingart *et al.*, 2000). Immunization of mice with CyaA protects against lethal challenge with *B. pertussis* (Guiso *et al.*, 1989) and increases the rate of bacterial clearance after sub-lethal infection (Guiso *et al.*, 1991). Inhibition of anti-bacterial functions, including chemotaxis, phagocytosis and superoxide generation, in host immune cells has been shown to be a result of the formation of supraphysiological levels of cAMP by CyaA (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987). CyaA has been shown to form pores and cause lysis and cytotoxicity in a variety of cells including macrophages (Khelef *et al.*, 2001), cardiac myocytes (Otero *et al.*, 1995), red blood cells (Westrop *et al.*, 1997) and T cells (Lee *et al.*, 1999). CyaA has also been shown to induce apoptosis of macrophages in *vitro* (Khelef and Guiso, 1995, Khelef *et al.*, 1993) and *in vivo* (Gueirard *et al.*, 1998). These results demonstrate that CyaA is an important virulence factor of *B. pertussis* and plays a role in immune subversion and modulation of the immune response by the bacteria.

1.5.5 Adenylate cyclase toxin targets and modulates innate immune cells

CyaA has been shown to bind several cell types in a receptor independent manner (Rogel and Hanski, 1992), but has most recently been shown to bind with high specificity to the surface expressed CD11b/CD18 $\alpha_M\beta 2$ integrin. This binding was shown to be saturable and could be completely blocked by monoclonal antibodies against CD11b (Guermonprez *et al.*, 2001). This surface molecule, also known as complement receptor-3 (CR3), is found primarily on leukocytes, including natural killer (NK) cells, neutrophils, a subset of $CD8^+$ cells, macrophages and DC (Arnaout, 1990, Bell *et al.*, 1999). The blocking of CD11b was shown to dramatically reduce the translocation of the catalytic domain into the cytosol of the target cells and

consequently prevent the production of cAMP, which demonstrates that the interaction of CyaA with its receptor is essential for optimal CyaA activity. In addition, calcium dependent conformational changes also shown to be required for interaction of CyaA with CD11b/CD18 (Guermonprez *et al.*, 2001). It has also been demonstrated that CyaA interacts with the greatest affinity with the CD11b subunit of the CR3 heterodimer, and more efficiently binds cells expressing this surface receptor (El-Azami-El-Idrissi *et al.*, 2003). Using truncation studies it was shown that this binding is mediated by residues 1166-1281 of the RTX domain of CyaA, demonstrating that the catalytic N-terminal domain does not play a role in the interaction of the CyaA protein with the target cell (El-Azami-El-Idrissi *et al.*, 2003). This had previously been suggested in a study that used truncations of the N-terminal of CyaA together with N-terminal specific antibodies that prevented translocation of the catylitic domain across the eukaryotic target cell membrane (Gray *et al.*, 2001).

The study by El-Azami-El-Idrissi *et al.* (2003) also showed that non-acylated CyaA retained limited ability to bind cells expressing CD11b, although at a much reduced efficiency, demonstrating that acylation of Lys^{983} is involved in receptor mediated cell binding by CyaA. Furthermore, the lack of acylation reportedly abolished the ability of CyaA to increase cAMP levels in target cells (El-Azami-El-Idrissi *et al.*, 2003).

CyaA plays a role in the activation of innate immunity and the activity of DC. Importantly, CyaA has been shown to target DC *in vivo* through its specificity for the CR3 integrin expressed on the surface of DC (Guermonprez *et al.*, 2002). It has also been demonstrated that CyaA modulates the activities of human monocyte derived DC, upregulating surface markers associated with maturation and suppressing inflammatory cytokine production (Bagley *et al.*, 2002b). It has been suggested that the sustained elevation of intracellular cAMP levels is responsible for the toxic effects of CyaA on target cells (Rogel *et al.*, 1991) and also that the activation of innate immune cells is due solely to this supraphysiological levels of cAMP (Bagley *et al.*, 2002b). It has also been suggested that the biological action of CyaA on target cells is entirely dependent on the posttranslational acylation of Lys^{983} by CyaC and that without this modification CyaA is unable to intoxicate either innate cells or red blood cells (Barry *et al.*, 1991,

Basar *et al.*, 2001, Hackett *et al.*, 1994). However, CR3 is the specific receptor for CyaA and non-acylated CyaA retains the ability to bind cells through this surface expressed integrin (El-Azami-El-Idrissi *et al.*, 2003). CR3 plays an important role in leukocyte adhesion by acting as a ligand for ICAM-I, fibrinogen, and components of the complement system (Altieri and Edgington, 1988, Beller *et al.*, 1982, Diamond *et al.*, 1990) and is an important mediator of intracellular signalling involved in cytoskeleton rearrangement, and phagocytosis (Agramonte-Hevia *et al.*, 2002). It is possible that binding of CyaA to CR3 may activate some intracellular signalling processes, but this has yet to be addressed.

1.5.6 Activation of acquired immunity by adenylate cyclase toxin

CyaA has also been implicated in the regulation of adaptive immunity; CyaA enhanced the production of antigen-specific antibodies when co-administered with a foreign antigen in mice (Hormozi *et al.*, 1999). The invasive nature of CyaA has also been employed to deliver many antigenic peptides to the endogenous route of antigen processing for MHC class-I restricted presentation to $CD8^+$ T cells (Simsova *et al.*, 2004). Recombinant CyaA proteins have been genetically constructed to include foreign T cell epitopes in the N-terminal domain. These epitopes are inserted along with the N-terminal domain of CyaA through the plasma membrane following the interaction of the recombinant CyaA proteins with the target eukaryotic cell. It has been shown that the C-terminal domain of CyaA does not reach the cytosol (Osicka *et al.*, 2000). This method of epitope delivery has been used to elicit a $CD8^+$ T cell response against heterologous foreign epitopes *in vivo* (Fayolle *et al.*, 1996) and has been shown to confer protective $CD8^+$ T cell mediated immunity against viral (Saron *et al.*, 1997) and tumor (Fayolle *et al.*, 1999) challenge *in vivo* and promote the induction of *Mycobacterium tuberculosis* specific T cells (Wilkinson *et al.*, 2005). In these studies CyaA acted as a vector to deliver the foreign $CD8^+$ epitope to the APC and assessment of immune responses focused on MHC class-I restricted cytotoxic T lymphocyte (CTL) activity. However, $CD4^+$ T cell help is required to activate the CTL response suggesting the T cell may also be activated. The N-terminal does not need to be enzymatically

active to reach the cytosol (Iwaki *et al.*, 1995, Sebo *et al.*, 1999) and in many studies the catalytic site was genetically ablated to eliminate any potential cytotoxic effects associated with the up-regulated cAMP levels (Steinman, 1991). These detoxified CyaA molecules function as effective delivery systems for antigens and epitopes (Dadaglio *et al.*, 2003, Fayolle *et al.*, 1999, Fayolle *et al.*, 2001, Loucka *et al.*, 2002, Mascarell *et al.*, 2005, Sebo *et al.*, 1999, Wilkinson *et al.*, 2005).

While it has been shown that a detoxified CyaA could deliver an epitope into the MHC class-II presentation pathway, required for presentation to and activation of $CD4^+$ cells (Loucka *et al.*, 2002), both $CD8^+$ T cells and $CD4^+$ T cells have been detected following delivery of epitopes and whole antigens from *M. tuberculosis* (Wilkinson *et al.*, 2005), HIV (Mascarell *et al.*, 2005), lymphocytic choriomeningitis virus (LCMV) (Dadaglio *et al.*, 2000) and the model antigen OVA (Schlecht *et al.*, 2004) by CyaA molecules. The $CD4^+$ T cell response was characterised as Th1-baised based on IFN-γ secretion. This $CD4^+$ response produced by CyaA bearing the antigens was dependant upon the binding of CD11b by the CyaA carrier molecule (Schlecht *et al.*, 2004) and MHC class-II processing and presentation (Wilkinson *et al.*, 2005). Many of these studies included alum with the formulation of CyaA toxoids (Dadaglio *et al.*, 2003, Dadaglio *et al.*, 2000, Mascarell *et al.*, 2005). While alum is a potent adjuvant, its mechanism of action is not yet fully understood. However, it has been shown to enhance humoral immunity and the induction of Th2 cells (Brewer *et al.*, 1999). The presence of alum in these studies complicates the interpretation of the data and the understanding of the roles played by CyaA, the delivered antigen and alum in those experiments.

1.6 Transcriptional analysis of immunomodulation by pathogen derived molecules

Microarray technology is built upon a foundation of traditional molecular biology and allows the expression patterns of thousands of genes to be compared simultaneously. In basic research the strength of this technique is in finding differences in gene expression patterns across samples, such as differences between diseased and healthy tissues, differences between untreated cells and cell treated with drugs or toxins and changes in gene expression over time in cancer progression analysis (Butte, 2002,

Stears *et al.*, 2003). Complimentary (c)DNA microarrays (Schena *et al.*, 1995) and oligonucleotide microarrays (Lockhart *et al.*, 1996) are grids of nucleic acid fabricated in such a way so that each element of the grid probes for a specific RNA sequence, each coding for a sequence that is the reverse compliment of the target RNA. Both variations on the technology involve extracting RNA, which may then be further purified to messenger RNA (mRNA). The RNA is then copied and labeled with a florescent marker or other tag that can later be stained with a florescent marker. This labelled RNA is then hybridized to a microarray, and is washed free of excess RNA after the incubation period and then scanned by a light laser that allows the intensities of the fluorescence to be correlated to the level of expression of a particular gene. Two types of technology for hybridization are widely used. The first of which is single colour analysis (Fig. 1.5a) whereby the RNA from control (untreated) and experimental (treated) samples are purified and labelled with the same fluorescent label and each is then hybridized to a separate microarray. Each of these microarrays are then read separately by laser and the images from each chip are compared by computer algorithms. The data gives an absolute measurement of each RNA transcript present, which must then be compared by computer with that of another array for analysis. Two-colour analysis (Fig. 1.5b) involves labelling the control and experimental sample with different fluorescent markers and hybridizing them to the same array, which is then read with two lasers of different wavelengths. This has the advantage of being able to directly compare two samples. Regardless of the technology, the end result is a measurement of the changes in up to 50,000 gene transcription levels in a single sample. The technology has progressed to such an extent that the rate limiting step is not the handling of the biological samples or the actual array analysis, but is the post analytical work to determine the significance and function of the regulated genes (Brazma *et al.*, 2001). The "list of genes" generated by microarray analysis can not be viewed as a result in itself but must be interpreted in terms of the experimental conditions. Although an experimenter does not necessarily need to have a set of interesting candidate genes in mind before beginning the analysis, the biological importance of the genes whose expression levels are changed to the greatest extent must be evaluated in terms of their function. Unfortunately names and functions for

these genes may not be available, merely a sequence and an annotation deposited in a database. Additionally, many gene functions have been assigned based on homology to known genes and proteins from different systems or species, and as such for complete biological significance to be elucidated further validation must take place (Butte, 2002).

Microarrays have been applied to immunological studies to investigate the changes in gene expression in response to infection or induced by pathogen-derived molecules. For example, changes in gene transcription in innate cells in response to *Mycobacterium tuberculosis* (Ragno *et al.*, 2001), *Salmonella dublin*, (Detweiler *et al.*, 2001, Eckmann *et al.*, 2000) and *B. pertussis* (Belcher *et al.*, 2000) infections have been investigated. Other studies have compared the changes in gene expression in innate cells due to infection with different pathogens (Boldrick *et al.*, 2002, Huang *et al.*, 2001, Nau *et al.*, 2002). The study by Huang *et al.* (2001) identified a pool of 166 common genes modulated by all three organisms studied, and further identified groups of genes modulated only by individual pathogens. There have been few published studies that have used microarrays to investigate the modulation of immunity by purified pathogen-derived molecules. Nau *et al.* (2002) have demonstrated that the early responses to *M. tuberculosis* are mediated through stimulation of TLR2 and TLR4. However, the TLR ligands studied did not reveal a mechanism for the suppression of IL-12 production by the bacteria. Studies by Huang *et al.* (2001) also identified genes whose expression was modulated by LPS, mannose and double stranded RNA. These studies demonstrate that microarray technology can provide important insights into the mechanisms by which pathogens modulate the immune system during infection and also how pathogen derived molecules affect immune cells. Such data not only allows investigation of how pathogens modulate the immune system, which is important in designing new treatments and vaccines, but also adds to the understanding of the mechanism of activating immunity against infectious agents.

1.7 Aims

The aims of these studies are to investigate how the adenylate cyclase toxin of *B. pertussis* modulates the immune system and to understand how this modulation is mediated through innate and adaptive immune responses. To better understand how

CyaA affects innate immune cells, microarray technology is used to investigate how CyaA modulates gene transcription at a global level. In addition, the roles of the enzymatic domain and post-translational acylation of CyaA in immunomodulation are investigated. The potential use of CyaA as a vaccine adjuvant and immunotherapeutic molecule is also considered in these studies.

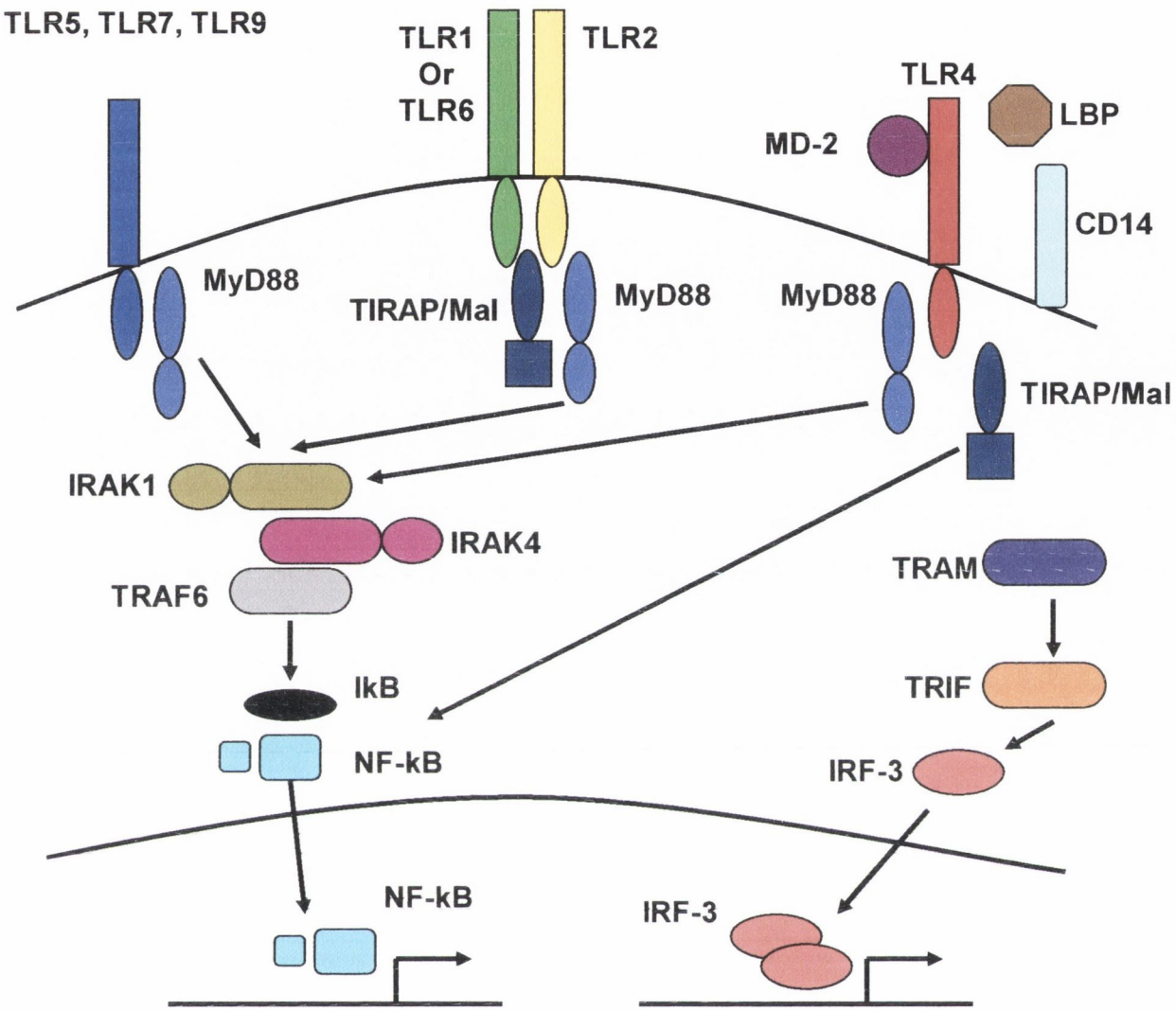


Figure 1.1 Schematic of the Toll-like receptor (TLR) signaling pathways.

TLR molecules are depicted together with their associated adaptor molecules which recruit additional intracellular molecules and and the activation of a kinase cascade, which activates transcription factors that translate to across the nuclear membrane, bind DNA and activate response genes.

Figure adapted from Akira, S. 2003. *J Biol Chem* 278: 38105-38108 and Akira and Hemmi,(2003) Immunol *Lett* 85:85-95.

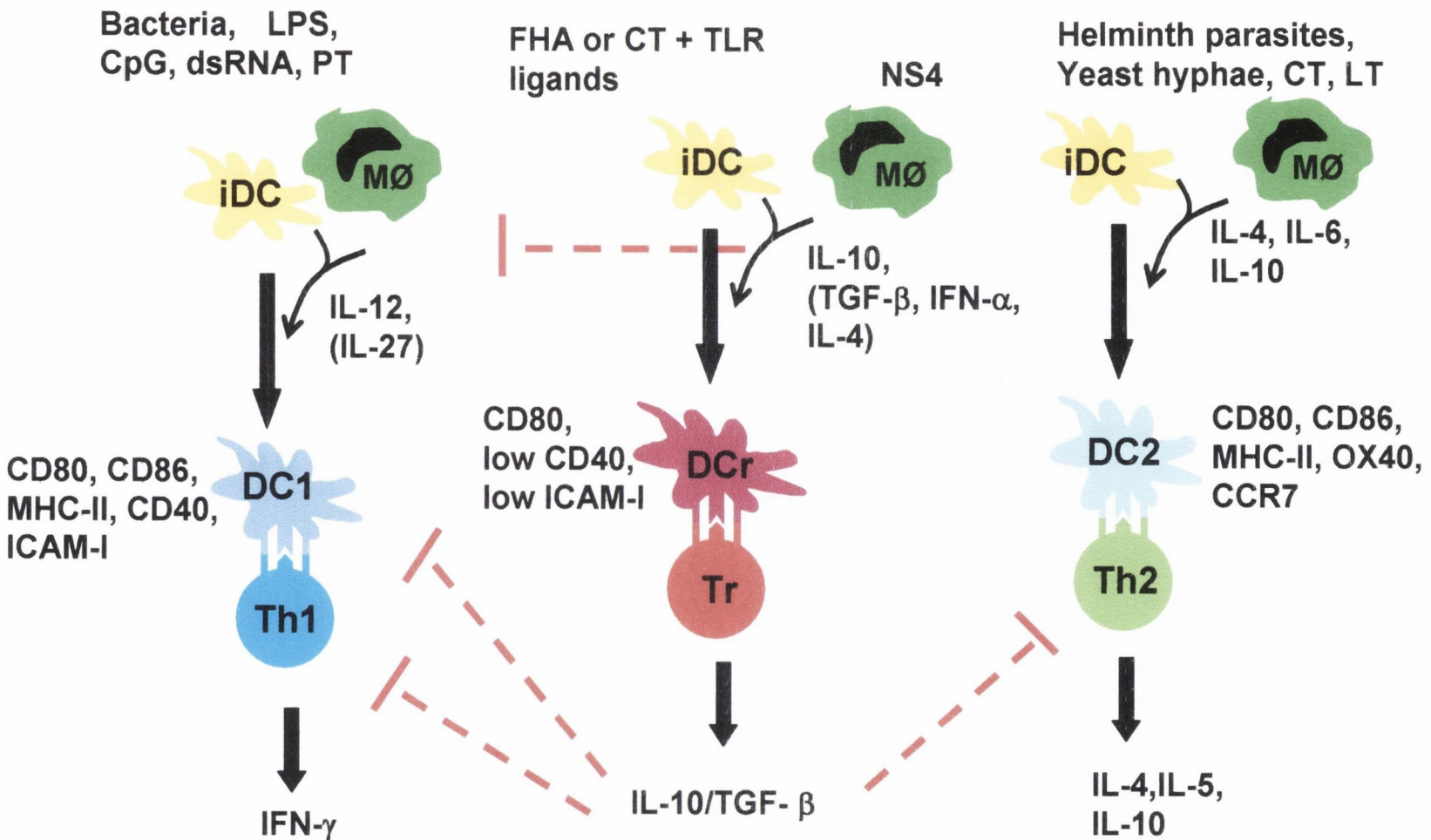


Figure 1.2 Proposed model for $CD4^+$ T-cell differentiation and polarization by mature and activated DC.

Certain pathogen-derived immunomodulatory molecules bind to dendritic cells (DCs) or other innate cells, including macrophages, and stimulate maturation of immature DCs (iDCs) into DC1 and DC2, which direct the differentiation of Th1 and Th2 cells, respectively. Other pathogen-derived molecules activate and mature DC to a phenotype (DCr) that directs the induction of regulatory T (Tr) cells. Tr cells suppress the activity of Th1, and possibly Th2, cells by secreting regulatory cytokines and may also act directly on the antigen presenting cell through IL-10 secretion.

Modified from work presented by the Immune Regulation Research Group, School of Biochemistry and Immunology, Trinity College Dublin.

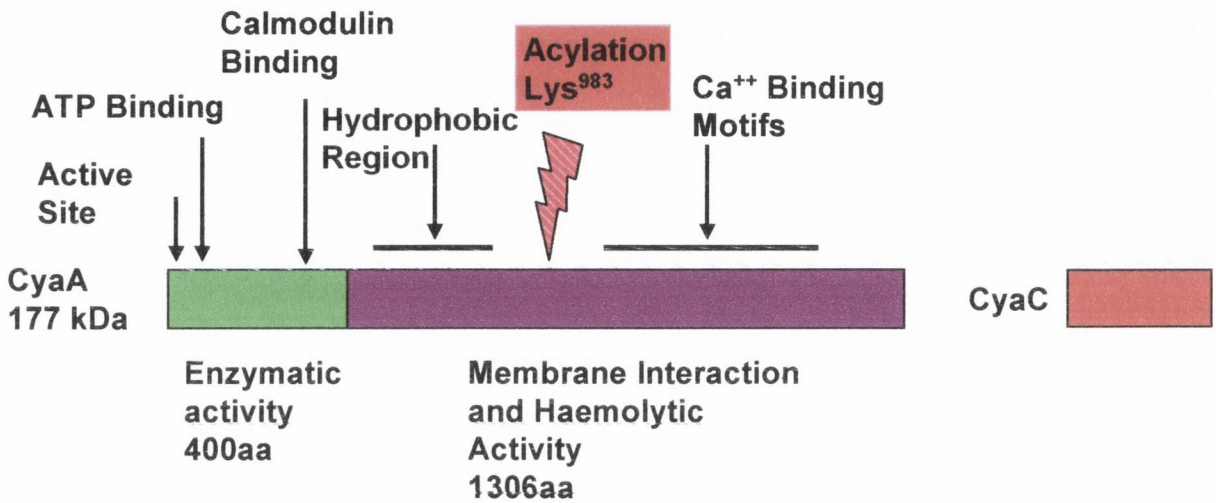


Figure 1.3 Structure of adenylate cyclase toxin (CyaA) of *Bordetella pertussis*.

The enzymatic domain of CyaA in the N-terminal 400 amino acids contain the active site, which converts ATP to cAMP. The catalytic domain of CyaA requires binding of eukaryotic calmodulin to become fully active. The catalytic domain converts ATP to cAMP and is dependant upon binding to eukaryotic calmodulin. Interaction with the eukaryotic cell membrane occurs via the C-teminal hydrophobic domain and is dependant on the binding of calcium ions. This binding results in the formation of a pore in the cell membrane through which the N-terminal enzymatic domain is inserted directly into the cytosol of the target cell and through which potassium ions escape. Lysine^{983} is acylated by the specific accessory protein, CyaC. This post-translational modification plays a role in some of the activities of CyaA.

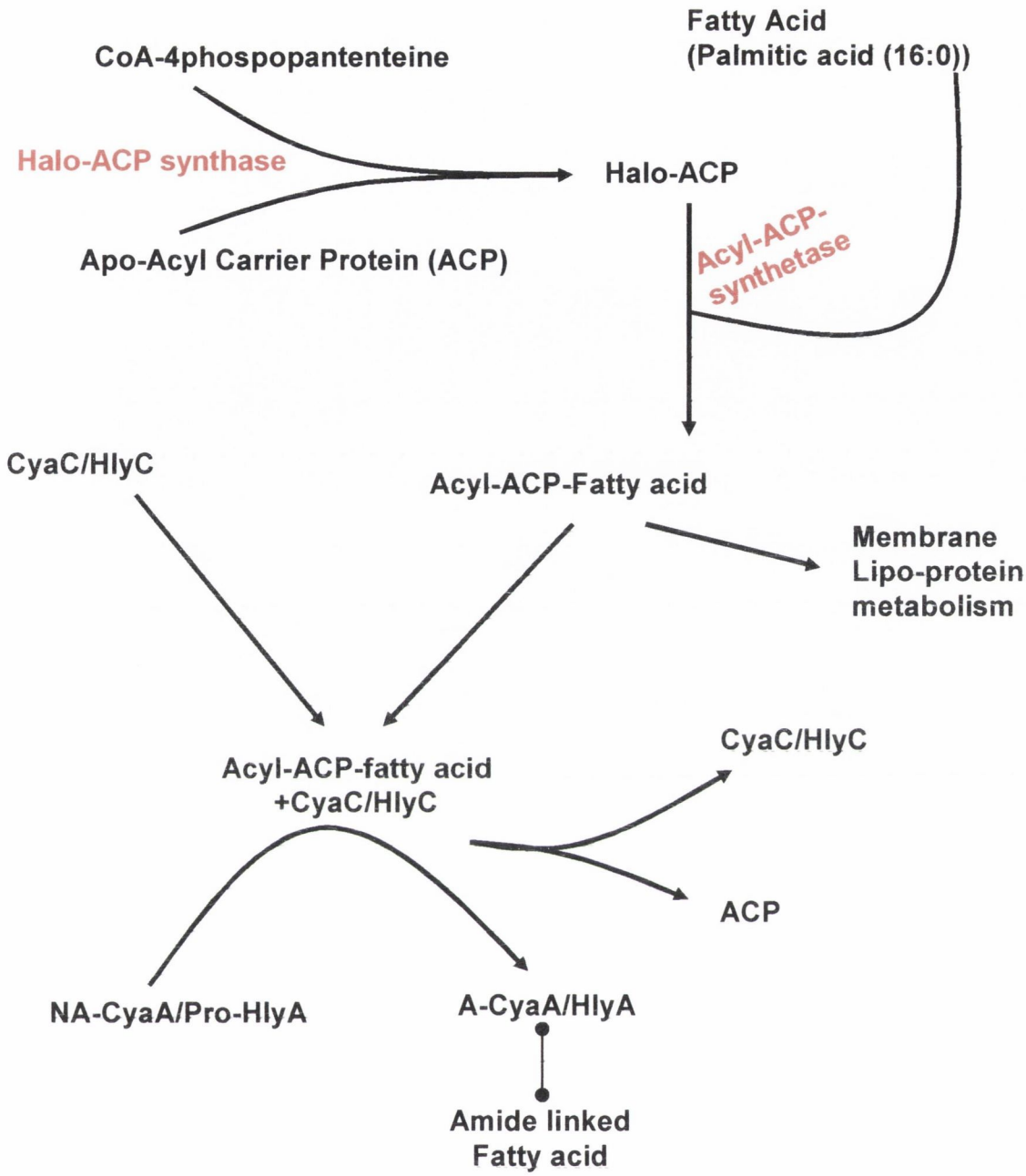


Figure 1.4 Fatty acid acylation of CyaA and HlyA
Proposed mechanism of palmitic acid fatty acid acylation of *B. pertussis* CyaA by CyaC and *E. coli* HlyA by HlyC.

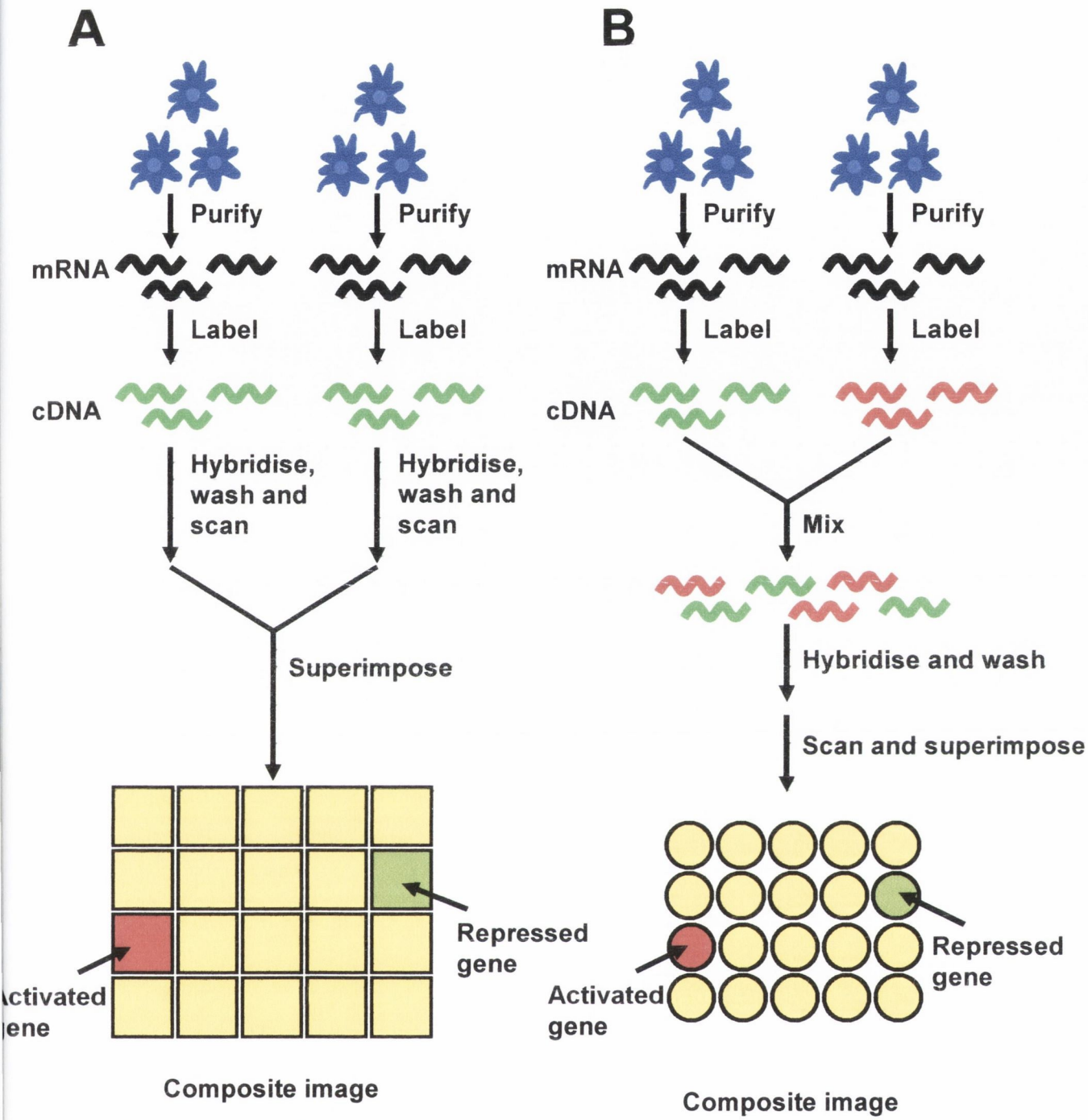


Figure 1.5 RNA expression analysis by microarray.
a) One-color expression analysis uses a single fluorescent label (green wavy lines) and two chips to generate expression profiles for two or more cell samples. Activated and repressed genes (green and red squares, respectively) are obtained by superimposing images obtained from different chips. *b*) Two-color expression analysis uses two different fluorescent labels (green and red wavy lines) and a single chip to generate expression profiles for two different cell samples. Activated and repressed genes (red and green spots, respectively) are obtained by superimposing images generated in different channels on a single microarray. Genes expressed equally in the two samples appear as yellow squares or spots in the two analyses.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media and buffers and reagents

2.1.1 Chemicals

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1.2 Tris, Boric acid, EDTA (TBA)

A stock of 10X TBE was prepared by dissolving 107.8 g Tris Base, 50 g Boric acid and 7.44 g EDTA in 1 litre of dH_2O, This was then adjusted to pH 8.3 and a 1X working solution prepared for use in experiments.

2.1.3 DNA Loading-dye

A stock of 6X DNA loading-dye was prepared in dH_2O, typically in a volume of 10 ml and stored at room temperature before use and contained 10 mM Tris-HCl, 50 mM EDTA, 50% (w/v) sucrose, 0.25% (w/v) bromophenol blue.

2.1.4 Hanahan's broth (SOB media)

To prepare SOB Media , the following were dissolved in 1 litre of dH_2O, 20g Bacto tyryptone (BD Difco, MD, USA), 5g Yeast extract (BD Difco, MD, USA), 0.584g NaCl and 0.186g KCl. It was the adjusted to pH 7.0 and autoclaved to sterilise.

2.1.5 SOC media

To prepare SOC media, 100 µl 2M glucose and 100 µl 2M Mg^{++} were filter sterilised and added to 10 ml of autoclave sterilised SOB media. This was prepared on the day of use and could be stored for up to three days.

2.1.6 Luria Bertani broth (LB)

Luria Bertani broth (LB) was prepared by dissolving 10 g Tryptone, 5 g yeast extract and 10 g NaCl in 1 litre dH_2O. This was then adjusted to pH 7.0 and autoclaved to sterilise. Alternatively, a commercial mixture (BD Difco, MD, USA) was purchased and made up according to the manufacturers instructions and autoclaved to sterilise.

2.1.7 LB-agar

For the growth of *E. coli* on plates, LB broth was solidified by the addition of agar (BD Difco, MD, USA; 15 g/litre) prior to autoclaving. Where necessary, antibiotics were added when cooled and this was plated into 10 cm Petri dishes taking care not to introduce any air bubbles, and allowed to cool and harden. This could be stored at 4°C for 1 week before use.

2.1.8 Stainer and Scholte Medium

Basal Medium

10.72 g L- Glutamic acid (monosodium salts)

0.24 g L-Proline

2.5 g NaCl

0.5 g KH_2PO_4

0.2 g KCL

0.3 g Magnesium Chloride ($MgCl_2.6H_2O$)

0.4 g Calcium Chloride ($CaCl_2.2H_2O$)

1.525 g Tris

Dissolved in 1L of ddH_2O and ph adjusted to 7.2. The medium was then autoclaved at 121°c for 20 mins, allowed to cool and 1 ml of supplement added per 100 ml of basal medium before use.

Supplement

0.4 g L-Cysteine (dissolved in 1 ml of concentrated HCL)

0.5 g Ferrous Sulphate ($FeSO_4.7H_2O$)

0.6 g Ascorbic Acid

0.7 g Nicotinic Acid

1.0 g Glutathione

Dissolved in 100 ml of dd H_2O, filter sterilized and stored at 4°C for up to 5 weeks.

2.1.9 Foetal calf serum (FCS)

Foetal calf serum (FCS) was purchased from Biosera (East Sussex, UK) and was heat inactivated at 56°C for 30 minutes prior to use. The batch of FCS (batch number

S04304S1810) used throughout these experiments was tested and was found to have 20 pg LPS/ml.

2.1.10 Complete Roswell Park Memorial Institute-1640 media (cRPMI)

Roswell Park Memorial Institute (RPMI)-1640 media was purchased from Biosera (East Sussex, UK), This was tested and found not to have detectable levels of contaminating LPS. Before use, RMPI-1640 was supplemented with 8% heat-inactivated FCS, 100 mM L-Glutamine (Gibco, NY, USA), 100 μg/ml streptomycin (Gibco, NY, USA) and was termed complete RPMI (cRPMI). The final concentration of contaminating LPS was 1.45 pg/ml.

2.1.11 Complete Duelbeccos modified eagles media (cDMEM)

Duelbeccos modified eagles media (DMEM) was purchased from Sigma (Poole, UK), This was tested and found not to have detectable levels of contaminating LPS. Before use DMEM was supplemented with 8% (v/v) heat-inactivated FCS, 100 mM L-Glutamine (Gibco, NY, USA), 100 μg/ml streptomycin (Gibco, NY, USA). The final concentration of contaminating LPS was 1.45 pg/ml.

2.1.12 Phosphate buffered saline (PBS)

A 1X solution of phosphate buffered saline (PBS) was prepared by dissolving 80 g sodium chloride (NaCl), 11.6 g sodium hydrogen phosphate (Na_2HPO_4), 2 g Potassium dihydrogen phosphate (KH_2PO_4), 2 g Potassium chloride (KCl) in 1 litre of dH_2O which was then adjusted to pH 7.0. A 20X stock was prepared and diluted to a 1X working concentration with dH_2O prior to use. Sterile Dulbecco's PBS was purchased from Biowest (Nuaillé, France), which was tested and found not to have detectable levels of contaminating LPS and was used in both *in vitro* and *in vivo* immunomodulation experiments and in protein purification protocols.

2.1.13 PBS-Tween (PBS-T)

Tween 20 is a commercial surfactant and 0.05% (v/v) was added to 1X PBS to make wash buffer for use in ELISAs and Western blots.

2.1.14 Phosphate citrate buffer o-phenylendiamine (OPD) ELISA substrate

Phosphate citrate buffer is used as the diluent for the o-phenylendiamine (OPD) ELISA substrate. Phosphate citrate buffer was prepared by dissolving 10.19 g citric acid monohydrate and 36.9 g sodium hydrogen phosphate in 1 litre dH_2O. This was adjusted to pH 5 and stored at 4°C prior to use. OPD substrate was purchased from Sigma Aldrich (Dublin, Ireland) in pre-prepared 10 mg tablets. Each 10 mg OPD tablet was dissolved in 25 ml of phosphate citrate puffer, pH5, and prior to use was activated by the addition of 16 µl of hydrogen peroxide (H_2O_2).

2.1.15 3,3',5,5'-Tetramethylbenzidine (TMB)

3,3',5,5'-Tetramethylbenzidine (TMB) is the horseradish peroxidase (HRP) substrate used for commercial ELISA kits. It was purchased from Sigma-Aldrich (Dublin, Ireland) in a ready to use form and stored at 4°C before use.

2.1.16 Polymyxin B

Polymyxin B sulphate was purchased from Sigma (Poole, UK) as a dry powder. A stock solution at a concentration of 10 mg/ml in dH_2O was stored at 4°C. Polymyxin B (PB) chelates endotoxin and was used to negate the effect of the low levels of any LPS in the preparations of the recombinant toxins. Polymyxin B was used at a concentration of 10 µg/ml in *in vitro* experiments where necessary.

2.1.17 Ammonium chloride lysis solution

Ammonium chloride lysis solution was used to lyse red blood cells in *ex vivo* cell preparations. This was prepared by dissolving 0.87 g Ammonium chloride (NH_4Cl) in 100 ml dH_2O and adjusted to pH 7.2. The solution was filter sterilized before use.

2.1.18 Trypan blue

A 0.4% (w/v) solution of trypan blue stain was purchased from Sigma-Aldrich (Dublin, Ireland). Trypan blue was used to ascess cell viability and concentration. Cells were typically diluted 1 in 10 with trypan blue and observed on a haemocytometer under a

light microscope. Live cells exclude the blue dye and appear white, while dead and dying cells appear blue.

2.1.19 PBS, BSA, Azide (PBA)

PBA was the buffer used in the preparation of cells for analysis by flow cytometry. It is prepared by adding 0.5% w/v BSA (Sigma, Dublin, Ireland) and 0.02% (w/v) azide (NaN_3) to PBS. Typically, this is prepared the day before use and allowed to mix over night at 4°C. NaN_3 is very toxic and usually diluted from a stock of 2% (w/v) in dH_2O.

2.1.20 2X SDS-PAGE loading dye

2x SDS-PAGE loading dye was prepared in dH_2O and contained the following, 10 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2 % (w/v) Bromophenol Blue, 20% (v/v) glycerol. On the day of use, 200 mM DTT was added.

2.1.21 SDS-PAGE running buffer

A stock of 5X SDS-PAGE Running buffer was prepared in dH_2O by adding the following, 124 mM Tris-Base, 960 mM Glycine, 17 mM SDS and it was adjusted to pH 8.6. This was diluted to a 1X working concentration on the day of use.

2.1.22 SDS-PAGE transfer buffer

A stock of 10X SDS-PAGE Western blot transfer buffer was prepared in dH_2O by adding 48 mM Tris-Base and 390 mM Glycine. This was diluted to a 1X working concentration on the day of use.

2.2 Bacterial Strains

2.2.1 *Bordetella pertussis*

Bordetella pertussis Wellcome 28 phase 1 (W28) stocks were provided by Dr. Keith Readhead at the National Institute for Biological Standards and Control (NIBSC).

2.2.2 *Escherichia. coli*

The *Escherichia coli* XL1-blue (K12) strain was used for all molecular cloning and protein expression experiments. A stock was purchased from Stratagene (La Jolla, CA, USA) by Declan Madsen of the Molecular Biology laboratory NUI Maynooth, Co. Kildare, from whom glycerol stocks were obtained.

2.3 Genetic manipulations and molecular cloning

All molecular cloning and genetic transformation was performed according to standard techniques described by Sambrook *et al.* (Sambrook and Russell, 2001).

2.3.1 Growth of *E. coli*

E. coli were used as the host strain for plasmids and protein recombinant protein expression. *E. coli* from frozen glycerol stocks were grown by streaking a small amount of frozen bacteria with an inoculating loop onto an LB plate containing the appropriate antibiotics. This was incubated overnight at 37°C and single colonies were recovered the following morning. Liquid cultures of *E. coli* were grown in LB broth with antibiotics where needed. These were seeded with a single colony from an LB plate and grown with vigorous shaking at 37°C.

2.3.2 Preparation of *E. coli* electro-competent cells

A 5 ml culture of *E. coli* was grown from a single colony in SOB overnight. 3 ml of this was used to inoculate 200 ml of fresh SOB the following morning and this was grown to mid-log phase (OD_{600}=0.5-0.8). The culture was cooled on ice for 30 minutes and centrifuged at 3200 rpm for 20 minutes at 4°C. The bacterial pellet was resuspended in the same volume (200 ml) of ice cold 10% glycerol. This was again centrifuged at 3200 rpm for 20 minutes at 4°C and the pellet resuspended in half the volume (100 ml) of 10% glycerol. This was again centrifuged at 3200 rpm for 20 minutes at 4°C and the pellet resuspended in 1/20 of the volume (10 ml) of 10% glycerol. The culture was centrifuged one final time at 5000 rpm at 4°C for 5 minutes and the pellet resuspended in 1/200 of the volume (1 ml) in 10% glycerol. These electro-competent *E. coli* cells were stored in 50 µl aliquots at -80°C until use.

2.3.3 Transformation of *E. coli* by electroporation

Aliquots of electro-competent cells (50 µl) were thawed from -80°C on ice, intact plasmid (typically 50 ng plasmid DNA) or DNA from a reaction to ligate an insert into a linearised plasmid (typically 10% of the resultant DNA) was added to the cells and

mixed by pipetting before transferring to a 0.1 cm electroporation cuvette (Biorad) which had been cooled on ice for 5 minutes. This was placed into an electrophoration machine at 1.8 kV with a time constant of 4-4.5 and pulsed. 700 µl SOC medium was then added to the culture and the 750 µl removed to a fresh 1.5 ml tube and incubated at 37°C with shaking for 1 hour. 100 µl was then plated at varying dilutions (typically undiluted, 1:10 and 1:100) on LB plates containing the appropriate antibiotic overnight at 37°C. Single colonies were then recovered and grown in liquid LB and the presence of the correct plasmid confirmed.

2.3.4 Glycerol stocks of *E. coli*

Glycerol stocks of *E. coli* were prepared and stored at -80°C to store the bacteria for long periods of time. To prepare, a 4 ml overnight *E. coli* culture was pelleted at 13,000 rpm for 2 minutes in a desktop microfuge. This was resuspended in an equal volume of fresh LB and pelleted again. Each culture was resuspended in a total volume of 1 ml LB to which was added 1 ml of 20% v/v glycerol/dH_2O, which had been filter sterilized. This was then aliquoted in two cryotubes and stored at -80°C.

Bacteria from glycerol stocks could be resurrected by scraping a small amount of frozen bacteria from the stock tube using an inoculating loop, streaking this on an LB agar plate containing the appropriate antibiotic and incubating at 37°C overnight. Glycerol stocks were never allowed to thaw as they cannot be refrozen.

2.3.5 Polymerase chain reaction (PCR)

PCR is a standard molecular cloning method for amplifying fragments of DNA using two oligodeoxynucleotide primers and DNA polymerase. The use of PCR was performed according to methods described by Sambrook *et al.* (Sambrook and Russell, 2001) although each template DNA and primer reaction was individually optimised, the general conditions for each reaction uses in the experiments below are detailed in Table 2.1. Taq DNA polymerase from *Thermus aquaticus* is used routinely in PCR reactions and retains DNA polymerase activity at high temperatures up to 80°C, although typically used at 72°C. Errors occur during the synthesis of each complementary strand of DNA, as high as $1x10^{-4}$ errors per base pair (bp) have been reported for Taq

polymerase (Barnes, 1992). Cloned genes used as templates for recombinant proteins must have their DNA correct sequence preserved. Pfu DNA polymerase isolated from *Pyrococcus furiosus* has the lowest error rate of routinely used DNA polymerases of roughly $1.3x10^{-6}$ per bp (Cline *et al.*, 1996). For this reason, Pfu DNA polymerase was used to clone genes from genomic DNA and in addition the sequence of all cloned genes was confirmed by DNA sequencing.

2.3.6 Visualisation of DNA following electrophoresis

DNA fragments were visualised following electrophoresis on an agarose gel. Typically, DNA was separated on a 1 % w/v agarose gel in TBE for 45 minutes at 100 V. Samples were run along side an appropriate molecular weight marker of known sizes to aid identification of the DNA fragments. The gels were prepared with 30 μg/ml ethidium bromide, which binds to the DNA fragments in the gel and allows them to be visualized under a UV lamp. Plasmid DNA was visualized by this method, although it does not run on the gel at the same rate as linear DNA fragments. Instead, two bands of high molecular weight are usually observed after visualization of plasmid DNA corresponding to two forms of plasmid structure, super-coiled and open circle.

2.3.7 Purification of DNA fragments from agarose gels

Following a PCR reaction or digestion of plasmid DNA with restriction enzymes, the resulting fragments were separated and visualised as needed on a 1% agarose gel by electrophoresis. The band of interest was identified by comparison to a molecular weight marker run along side the experimental samples and was excised from the gel. These were stored at -20°C or purified immediately using the Concert Gel Extraction System (Gibco BRL, NY, USA) according to manufacturers instructions.

2.3.8 Digestion of plasmid vectors

Plasmid DNA can be digested with restriction enzymes to linearise the vector or open it at the multi-cloning site to allow the insertion of a DNA fragment amplified by PCR or a fragment of DNA from a plasmid. Typically, 2 μl of midi-prep plasmid DNA was digested in each reaction overnight at 37°C. It is possible to digest a plasmid or PCR

fragment simultaneously with two restriction enzymes, providing that the restriction sites are suitably far apart to allow both enzymes access to their respective sites and that the reaction buffers are compatible for both enzymes.

2.3.9 Calf intestinal alkaline phosphatase (CIAP) treatment of vectors

Plasmid vectors that were digested with restriction enzymes were always treated with calf intestinal alkaline phosphatase (CIAP) to remove the phosphate on the 5'-overhang to prevent re-ligation. Typically, following a digestion of a plasmid vector in a volume of 30 µl, 5 µl of 0.01 unit/µl CIAP and 5 µl of 5X CIAP buffer were added along with 10 µl dH_2O to bring the reaction to a volume of 50 µl. This was incubated at 37°C for 30 minutes at which time, another 5 µl of CIAP (0.01 unit/µl) was added and incubated for a further 30 minutes at 37°C. This could then be stored at -20°C before purification using the Concert Rapid PCR Purification System (Gibco BRL) according to manufacturers instructions.

2.3.10 Restriction digestion of PCR products.

PCR products that have been purified can be digested with restriction enzymes to allow their ligation into opened plasmid vectors. This is done in a similar manner to the digestion of plasmid vectors with restriction enzymes. It is possible to use two restriction enzymes simultaneously is possible if the reaction buffer conditions allow it. Digested PCR fragments were not treated with CIAP after digestion, as ligation would not be successful due to the lack of 5'-phosphate on the DNA. Following digestion, PCR products were purified using the Concert Rapid PCR Purification System (Gibco BRL) according to manufacturers instructions.

2.3.11 Ligation of DNA fragments into cloning and expression plasmids

Purified PCR products or DNA fragments with the appropriate overhangs can be ligated into a linearised plasmid digested with the appropriate restriction enzymes. Typically, a ratio of 1:3 of vector to insert, based on a fragment per fragment basis, is required for optimal ligation and successful transformant recovery. This ratio was determined by comparing the intensity of the linearised vector and insert on a 1%

agarose gel and factoring this by the molecular size of the DNA fragments. T4 DNA ligase (Gibco BRL, NY, USA) and the supplied buffers were used for all ligation reactions according to the manufacturers instructions. Ligation reactions were performed in a volume of 30 µl for 2 hours at 37°C. Following each ligation reaction, the DNA was precipitated by adding 1 µl glycogen (*Mytilus edulis;* 50 µg/ml), 10 µl potassium acetate (3.1 M), 200µl isopropanol and 70 µl dH_2O. This was incubated for at least 1 hour at -20°C before centrifugation at 13,000 rpm in a microfuge. The DNA pellet was washed in 200 µl 70% (v/v) ethanol (EtOH) and centrifuged again at 13,000 rpm for 10 minutes. The supernatant was discarded and the pellet allowed to air dry for 15-30 minutes before being resuspended in 10 µl dH_2O, which was then stored at -20°C or transformed into competent *E. coli* cells.

2.3.12 Mini-prep of plasmid DNA

Up to 10 µg of plasmid DNA can be recovered using the mini prep protocol. DNA recovered using the mini-prep protocol was used to check the correct construction of a plasmid. 1.5 ml of overnight *E. coli* culture was pelleted by centrifugation in a microfuge for 2 minutes. The pellet was resuspended in 150 ml solution I (50 mM Tris pH 8.0, 10 mM EDTA, 200 µg/ml RNase A, dH_2O) by pipetting. 150 µl of solution II (0.2 M NaOH, 1% w/v SDS, dH_2O) was then added and mixed by continuously inverting the tubes for 5 minutes at room temperature. 150 µl of solution III (3 M potassium acetate, 5 M glacial acetic acid, dH_2O) was added and the mixture was centrifuged at 13,000 rpm in a microfuge for 10 minutes. The supernatant containing the DNA was transferred to a new tube and 800 µl ice cold 100% EtOH was added. The tube was tapped several times to wash the DNA pellet and then incubated at -20°C for 1 hour. The DNA was pelleted again by centrifugation at 13,000 rpm in a microfuge for 10 minutes. The supernatant was poured off and the pellet washed in 200 µl 70% EtOH at room temperature. The samples were centrifuged at 13,000 rpm in a microfuge for 10 minutes, the supernatant was discarded and the pellet air dried at room temperature for 10 minutes. The plasmid DNA was resuspended in 50 µl dH_2O and stored at -20°C.

2.3.13 Midi-prep of plasmid DNA

Up to 100 μg of plasmid DNA can be purified using the midi prep protocol, which was performed using the Concert Plasmid Prep Kit (Gibco Brl, NY, USA) with modifications to manufacturers instructions. Briefly, 10 ml of equilibration buffer (E4) was added to each column. A starting culture of 15-25 ml *E. coli* was grown over night and pelleted at 3200 rpm. The bacterial cell pellet was resuspended in 4 ml of cell suspension buffer (E1), which had been supplemented according to the manufacturers instructions with RNase. Cell lysis solution E2 (4 ml) was then added and incubated at room temperature with mixing for 5 minutes. Neutralising solution E3 (4 ml) was then added and the tubes inverted 5 times to mix well. The mixture was then centrifuged at 13,000 rpm in a microfuge at room temperature for 10 minutes. The supernatant was then loaded onto the equilibrated column and allowed to run through. The column was washed twice with 10 ml wash buffer (E5) taking care not to allow the column to run dry. The plasmid DNA was eluted into a fresh tube with 5 ml of elution buffer (E6). Isopropanol (3.5 ml) was added to precipitate the plasmid DNA and this was then centrifuged at 13,000 rpm in a microfuge at 4°C for 5 minutes. The supernatant was discarded and the pellet appeared white in colour on the side of the tube. This was washed in 3 ml of 70% EtOH and centrifuged again at 13,000 rpm in a microfuge at 4°C for 5 minutes. The supernatant was discarded and the pellet allowed to air dry at room temperature. The plasmid DNA was resuspended in 200 μl dH_2O and stored at -20°C.

2.3.14 Confirmation of plasmid structure by restriction analysis

After single colonies of bacteria were recovered from LB-agar plates containing the appropriate antibiotic, it was important to confirm that they harboured the correct plasmid and were not simply spontaneous antibiotic resistant mutants and that the insert had been ligated into the plasmid in the correct orientation. This was done by preparing DNA from a liquid overnight culture of the bacteria and digested with restriction enzymes. Digesting with one enzyme linearised the plasmid and allowed visualisation of a single band on an agarose gel. The size of the linearised plasmid was equal to that of the vector plus the insert. Digestion with two enzymes, at one restriction site within the insert and another within the vector, provided another method of checking correct

orientation. Ideally such a digestion would yield two fragments of different size that could be easily identified on the gel. When matched to the expected sizes, the presence and orientation of the insert was confirmed.

2.3.15 Confirmation of plasmid structure by DNA sequencing

When genes are cloned from genomic DNA by PCR, or site directed mutagenesis is performed, it is possible that errors occur during the DNA polymerase reaction, even when the polymerase used has proof-reading ability. It was therefore necessary to check the sequence of all plasmids. DNA sequencing was performed by MWG Biotech (Germany) on all expression plasmids to confirm the correct orientation and sequence of the cloned genes.

2.3.16 Growth of *B. pertussis*

B. pertussis Wellcome 28 phase 1 was grown at 37°C under agitation conditions in Stainer-Scholte liquid medium from archived stocks prepared by Prof. Kingston Mills and Brian O'Gorman. Bacteria were grown over 48 hours to mid-log phase (OD_{600}=0.5-0.8) and were used in experiments at this point.

2.3.17 Isolation of genomic *B. pertussis* DNA

A culture of *B. pertussis* was grown from frozen stocks to mid-logarithmic phase, pelleted by centrifugation at 3000 rpm and stored at -20°C. Genomic DNA was prepared using the Aquapure Genomic DNA Kit (Biorad, CA, USA) according to manufacturers instructions. Briefly, $1x10^9$ bacterial cells were thawed to room temperature and resuspended in 300 µl of lysis solution. This was incubated at 80°C followed by the addition 1.5 ml of RNase A solution. Tubes were inverted 25 times to mix well and then incubated at 37°C for 45 minutes. Tubes were cooled to room temperature and 100 µl of the protein precipitation solution was added. The tubes were vortexed for 20 seconds and then centrifuged at 13,000 rpm for 3 minutes in a desktop microfuge. Supernatant was transferred to a new tube and 300 µl of isopropanol was added, the tubes were inverted 50 times to mix contents well before centrifugation at 13,000 rpm for 1 minute. The supernatant was discarded and the tube air-dried. 300 µl

of 70% EtOH was added to the pellet. The DNA was pelleted again by centrifugation at 13,000 rpm for 1 minute, the supernatant removed and the pellet air-dried for 15 minutes at room temperature. The DNA pellet was resuspended in 100 µl DNA hydration solution and incubated at 65°C for 45 minutes, tapping occasionally to mix. This was then aliquoted and stored at -20°C.

2.4 Detection of LPS

LPS concentrations in protein and other preparations such as media and FCS, were assayed using either of two comparable highly sensitive commercial colorimetric assay kits, purchased from Biowhittaker (MD, USA) and Associates of Cape Cod Inc. (MA, USA). These assays are based on the limulus amoebocyte lysate (LAL) assay. Assays were performed where indicated according to manufacturers instructions. LPS concentrations were determined by comparison to a standard curve prepared using highly purified LPS supplied with each kit.

2.5 Detection of cAMP

cAMP was measured the highly sensitive competitive ELISA kit purchased from Amersham Biosciences (UK). The samples were prepared as detailed in experiments and tested according to the manufacturers instructions.

2.6 Visualisation of proteins by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE)

Purified proteins or bacterial lysates can be separated by size by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). The 200 kDa CyaA proteins were visualised following separation on a 7% SDS-PAGE gel. A 7% SDS-PAGE gel was prepared by mixing 5 ml dH_20, 2.3 ml Bis-Acrylimide (10% w/v), 2.5 ml 1.5M Tris (pH 8.8), 100 µl 10% SDS, 100 µl 10% Ammonium Per-sulphate, and 10 µl Temed. This was loaded into a clean gel rig (Sigma E-5889) between two glass plates and allowed to set, with 1 ml methanol layered on top to ensure an even surface. Once set, the methanol was washed off with dH_2O and excess water removed. A stacking gel made up by mixing 2.52 ml dH_2O, 0.325 ml Bis-Acrylimide (10% w/v), 0.526 ml 0.5M Tris pH 6.8, 25 µl 10% SDS, 25 µl 10% Ammonium Per-sulphate, and 10 µl Temed, which was layered on top of the separating SDS-PAGE gel and a comb of appropriate size inserted. This was allowed to set before being put into place in the gel rig and 1X SDS-PAGE running buffer added. Samples were diluted 1 in 2 with 2X SDS-PAGE loading dye and loaded onto the gel. A commercial pre-stained protein ladder of known size was also run along side experimental samples to aid in the identification of

proteins. To separate the proteins, the samples were run for 1 hour at 30 Amperes (Amps) per gel with the voltage set to the maximum on a Biometra P25 powerpack (Biometra, Germany). To visualise the separated proteins, each gel was removed from the gel rig and washed in dH_20 for 15 minutes and then incubated in 5 ml Gel-Code Blue reagent (Peirce, IL, USA) for at least 1 hour and washed three times in dH_2O for 5 minutes.

2.7 Western blotting

Western blotting was used to confirm the identity of proteins using specific antibodies. Proteins were first separated by SDS-PAGE and the proteins transferred from the SDS-PAGE gel to nitrocellulose membrane using a semi-dry transfer apparatus (Biometra Fast Blot E35) at 225 mAmps for 75 minutes. The membranes were blocked to prevent non-specific binding by incubation overnight in 0.5% BSA/PBS and then washed in wash buffer. Membranes were then incubated with the appropriate primary antibodies specific for the protein of interest. In the case of recombinant CyaA, rabbit derived anti-CyaA antibody (gift from Erik Hewlett, University of Virginia, VA, USA) was diluted 1 in 5000 in 0.5% BSA/PBS and incubated with the membrane for 1 hour at room temperature. Alternatively, CyaA was identified using a commercial monoclonal anti-His-tag antibody (Santa Cruz Biotechnology Inc., CA, USA) at a concentration of 0.2 μg/ml in 0.5% BSA/PBS for 1 hour at room temperature. After incubation with the primary antibody, membranes were washed 6 times for 5 minutes in wash buffer and then incubated with the appropriate secondary antibody. To detect CyaA, membranes were incubated with a 1 in 25,000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma) in 0.5% BSA/PBS for 1 hour at room temperature. Membranes were washed again in wash buffer and the commercial Super Signal Chemiluminescent reagent (Peirce) was used according to manufacturers instructions to visualise the protein on photographic film.

2.8 Construction of expression plasmids

2.8.1 His-tagged acylated CyaA (A-CyaA)

To express and purify recombinant acylated CyaA from *E. coli,* plasmid pJR2 was constructed which encodes the *cyaA* and *cyaC* genes. Genomic DNA of *B. pertussis* (strain W28) was prepared from a mid-log culture. The 5' end of *cyaA* was amplified by PCR with oligonucleotides (MWG Biotech, Germany) PAB5 5'-CGCCGGTACCATGCAGCAATCGCATCAGGCT-3' and PAB6 5'-TGGTGAATTCGCTCTTGCCCG-3' (restriction sites underlined). The resulting product was digested with *Kpn*I and *Eco*RI (Invitrogen, CA, USA), inserted in corresponding sites of the cloning vector pBluescript SK- (Stratagene, CA, USA), and this plasmid was named pAPB4. The 3' end of *cyaA* was amplified by PCR from *B. pertussis* genomic DNA with oligonucleotides PAB7 5'-AAGAGCGAATTCACCACATTCGTCG-3' and PAB2 5'-CGCGGATCCTCAGCGCCAGTTGACAGCCA-3' (restriction sites underlined; a summery of oligonucleotides is presented in Table 2.5). The product was digested with *Eco*RI and *Bam*HI and ligated into pBluescript SK- at the same restriction sites. This plasmid was named pAPB5, and was then digested with *Eco*RI and *Bam*HI and the 3' *cyaA* fragment was subcloned into the corresponding sites of pAPB4 giving a full-length *cyaA* gene. This plasmid was named pAPB6. *cyaC* was amplified by PCR from the genomic DNA of *B. pertussis* with oligonucleotides PAB3 5'-CGCGGATCCGAGGGCATGTCATGCTTCCGTCCGCC-3' and PAB4 5'-CGCGGCGAAGCTTTCAGGCGGTGCCCCGGC-3' (restriction sites underlines). The PCR fragment was digested with *Bam*HI and *Hin*DIII and cloned into the pASK-IAB6 (IBA GmbH, Germany) expression vector opened with the same restriction enzymes. This new plasmid was termed pAPB1. The intact *cyaA* gene was isolated from pAPB6, digested with *Kpn*I and *Bam*HI, cloned into pAPB1 upstream of the *cyaC* gene using the *Kpn*I and *Bam*HI sites, and this plasmid was termed pAPB8. pAPB8 was digested with *Kpn*I and *Hin*DIII and the 5.9 kb product containing *cyaA* and *cyaC* was cloned into the commercial His-tagged vector pQE-80 (Qiagen, UK) opened at the same restriction sites. The sequence and orientation of the cloned genes were confirmed

by restriction digestion and sequencing (MWG Biotech). This plasmid was named pJR2 from which His-tagged palmitoylated CyaA could be expressed in *E. coli*.

2.8.2 His-tagged non-acylated (NA-) CyaA

To express and purify non-acylated CyaA from *E. coli*, plasmid pJR1 was constructed. The pJR1 plasmid encoded the *cyaA* gene in the absence of the *cyaC* gene. *cyaA* was subcloned as a *Kpn*I/*Bam*HI fragment from pAPB6 (pBluescript SK- containing *cyaA*, described above) into the plasmid pASK-IBA6 (IBA GmbH, Germany) to generate plasmid pAPB7. A *Kpn*I/*Hin*DIII fragment comprising *cyaA* from pAPB7 was then cloned into the corresponding sites of the pQE-80 plasmid (Qiagen) to generate pJR1 from which NA-CyaA was expressed in *E. coli*. The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech).

2.8.3 His-tagged acylated enzymatically inactive (iAC-) CyaA

To express and purify CyaA with an inactive adenylate cyclase domain (iAC-CyaA), the plasmid pNM2 was constructed which encodes the *cyaA* gene with three amino acid substitutions (His^{63} to Ala^{63}, Lys^{65} to Ala^{63} and Ser^{66} to Gly^{66}) in the enzymatic domain. These mutated amino acid residues have been shown to be necessary for enzymatic activity of the catalytic domain of CyaA (Drum *et al.*, 2002, Glaser *et al.*, 1989, Munier *et al.*, 1992). First, plasmid pAPB9 was constructed by inserting the 3.5 kb *Sst*I fragment encoding the 5' end of *cyaA* from pJR2 into the corresponding site in pBluescript SK-. Using pAPB9 as a template, a 0.4 kb PCR product was generated with oligonucleotides pUC Forward (CCCAGTCACGACGTTGTAAAACG) and PAB27 (CAACCCCCAATCGGA*TCC*C*GC*GGCG*GC*CACGCCCAATCCTTTG – introduced *Bam*HI site underlined, mutated bases in italics) and a 1.7 kb product with oligonucleotides PAB17 (CAAAGGATTGGGCGTG*GC*CGCC*GC*G*GGATCC*GATTGGGGGTTG – introduced *Bam*HI site underlined) and PAB29 (CGTAGATCTCCATGGGACTGAGC – *Nco*I site underlined). The former PCR product was digested with *Xba*I/*Bam*HI and the latter with *Bam*HI/*Nco*I and ligated with *Xba*I/*Nco*I-digested pAPB9 to create pNM1. The 2.5 kb *Cla*I/*Kpn*I fragment of pNM1 was inserted into the corresponding sites of pJR2 to

create pNM2. The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech). This plasmid encodes a H63A/K65A/S66G mutant of His-CyaA together with CyaC.

2.9 Purification of recombinant CyaA and CyaA derivatives

2.9.1 Purification of recombinant A-CyaA

E. coli XL-1 Blue harbouring plasmid pJR2 was induced to express CyaA and CyaC by the addition of isopropyl-β-thiogalactopyranoside (IPTG, Bioline, UK) to an exponentially growing bacterial culture in Luria-Bertani (LB) broth supplemented with 150 μg/ml ampicillin with vigorous shaking at 37^0C. The bacterial culture was centrifuged and the bacterial pellet resuspended in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8.0, supplemented with protease inhibitor cocktail (P-8465 Sigma, UK). Bacteria were disrupted with FastPrep Protein Blue beads (QbioGene, CA, USA) in a FastPrep machine at speed 6 for 20 sec. The insoluble material containing CyaA was separated by centrifugation, washed with 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2% Triton X-100, pH 8.0 and incubated in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 8 M urea, pH 8.0 (Buffer A) for 1 h at room temperature with stirring. The solubilised CyaA was collected following centrifugation. After addition of NaCl to a final concentration of 0.1 M, CyaA was loaded on a DEAE cellulose (Sigma) column equilibrated with Buffer A supplemented with 0.1 M NaCl and eluted with Buffer A supplemented with 0.2 M NaCl as previously described (Sakamoto *et al.*, 1992). The protein was further purified on Ni^{++} columns (Qiagen, UK) under denaturing conditions by pH adjustment as recommended by the manufacturers and eluted in 100 mM $NaHPO_4$, 10 mM Tris-HCl, 8 M urea, 0.2 mM $CaCl_2$, pH 4.5. LPS removal was attempted using Detoxigel endotoxin removal columns (polymyxin B conjugated columns, Pierce, IL, USA) following the manufacturer's protocols. LPS was dissociated from CyaA by dialysis first against Dulbecco's PBS (Sigma), 1 mM EDTA, 1 M urea, pH 4.6 and then against Dulbecco's PBS, 0.1 mM $CaCl_2$, 2 M urea pH 8.0. The purified protein was stored in aliquots at -20°C. LPS was measured by a colourimetric LAL assay (QCL-1000; Biowhittaker, MD, USA) and protein concentrations were determined by Bradford (Bio-Rad). Proteins were separated by Sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) and visualised with Coomassie Blue (GelCode Blue Stain Reagent, Pierce). Alternatively proteins were transferred to a nitrocellulose membrane following SDS-PAGE and probed with anti-His Tag antibodies (Santa Cruz

Biotechnologies) or anti-CyaA antibodies (gift from Erik Hewlett). The bands were visualised by incubation with secondary anti-rabbit IgG horseradish peroxidase conjugated antibodies (Sigma) and chemiluminescent supersignal detection system (Pierce).

2.9.2 Purification of recombinant NA-CyaA and iAC-CyaA

NA-CyaA and iAC-CyaA were expressed from *E. coli* XL1-blue harbouring either the pJR1 and pNM2 plasmids. N-terminal His-tagged proteins were purified from inclusion bodies by diethylaminoethyl–sepharose and Ni^{++}-agarose chromatography as described above in section 2.9.1. However, polymyxin B columns were not used to remove LPS from the protein preparations as the capacities of the columns were low and passage of A-CyaA over these columns was found to be ineffectual to remove LPS contamination. Instead LPS was removed from the CyaA preparation by dialysis, first against Dulbecco's PBS (Sigma), 1 mM EDTA, 1 M urea, pH 4.6 and then against Dulbecco's PBS, 0.1 mM $CaCl_2$, 2 M urea pH 8.0. LPS was measured by a highly sensitive colourimetric LAL assay (Associates of Cape Cod). The proteins were judged to be greater than 95% pure by Coomassie stained SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane following SDS-PAGE, probed with anti-His antibodies (Santa Cruz Biotechnologies, CA, USA) and anti-CyaA antibodies (kind gift from Erik Hewlett) and visualised by incubation with secondary anti-rabbit IgG horseradish peroxidase conjugated antibodies and chemiluminescence detection. Each protein was recognised by both antibodies. For direct comparisons, a preparation of A-CyaA was prepared by this method. These preparations were used in the experiments detailed in chapters 5, 6 and 7.

2.10 Characterisation of CyaA

2.10.1 Acylation of CyaA and CyaA derivatives.

The co-expression of *cyaC* with *cyaA* in *E. coli* produces a CyaA protein that is acylated with palmitoyl fatty acids at Lys^{983} and Lys^{860} (Hackett *et al.*, 1994, Hackett *et al.*, 1995). Expression of *cyaA* alone in the same system results in a CyaA protein devoid of this post-translational modification. To confirm the acylation status of the preparations of NA-CyaA, A-CyaA and iAC-CyaA modified methods of Jackowski & Rock (Jackowski and Rock, 1986) and Issartel et al (Issartel *et al.*, 1991) were employed. Specifically, *E. coli* (strain XL1-blue) harbouring the plasmids pJR1 (encoding *cyaA*) and pJR2 (encoding *cyaA* and *cyaC*) were streaked from glycerol stocks and grown on LB plates with ampicillin (150 μg/ml) overnight at 37°C. Single colonies were inoculated in a liquid 5 ml culture of LB+Amp and grown overnight with shaking at 37°C. This was then diluted to a 5 ml culture of OD_{600}=0.2 and was grown until OD_{600}=0.4-0.6. The bacteria from 1 ml of this culture were harvested by centrifugation at 800 g for 10 min in a desktop microfuge and resuspended in 1 ml LB broth with ampicillin (150 μg/ml), supplemented with 5 μCi of [^{14}C(U)]-palmitic acid (0.1 mCi/ml; Perkin Elmer, MA USA) by shaking vigorously at room temperature for 1 h. Protein expression was induced with 1 mM IPTG for 15 min. The cells were harvested and washed in fresh medium, pelleted and re-suspended in SDS-PAGE loading dye and the equivalent of 300 μl of bacterial culture was electrophoresed on a 7% SDS-PAGE gel, which was then stained with gel-code blue reagent (Pierce) to visualise the proteins and identify the CyaA protein band. Highly purified recombinant A-CyaA (not radioactively labelled; 2 μg) was also run on the same gel to identify the recombinant protein in the bacterial lysate. Fluorography was then used to visualise the radio-labelled palmitoylated proteins on the gels (Jackowski and Rock, 1986). Briefly, gels were soaked in 5 ml Amplify solution (Amersham Biosciences, UK) in the dark. Excess solution was removed with tissue and the gels placed between two pieces of cling-film. Labelled protein were visualised by exposing gels to photographic film.

2.10.2 Enzymatic activity of CyaA and CyaA derivatives

In vitro cell free enzymatic activity of CyaA was measured using the method previously described by Ladant *et al.* (Ladant *et al.*, 1986). Briefly, 0.1 μg protein was incubated for 5 min at 30°C in 50 μl 50 mM Tris-HCl, 100 μg/ml BSA, 0.1 μM calmodulin, 0.12 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM ATP, pH 8.0. The reaction was mixed with lysis reagent 1B of the Biotrak enzymeimmunoassay system (Amersham Biosciences, UK) and boiled for 5 min. cAMP was measured by a competitive ELISA in the Amersham kit. The concentration of cAMP was expressed as μmol cAMP per min at 30°C, pH 8.0. The ability of CyaA and CyaA derivatives to increase intracellular cAMP concentrations (sometimes called intoxication) was measured by culturing macrophages (J774 cell line) at $1x10^6$ cells/ml in DMEM medium with CyaA (1 μg/ml). After 30 min, 4 h and 24 h incubation, lysis reagent 1B of the (Amersham Biosciences, UK) was added and the samples processed for quantification by cAMP ELISA using the Biotrak Enzymeimmunoassay system and are expressed in pmol cAMP/10^6 cells.

2.10.3 Cell lysis assays

Toxin was added to J774 macrophages at the indicated concentrations. In certain experiments 10 μg/ml anti-mouse CD11b antibody (M1/70) or purified rat IgG_{2b}, κ isotype control (both from PharMingen), was added 30 min prior to the addition of CyaA and plates incubated at 37°C for 6 h. Cell lysis was measured by the release of lactate dehydrogenase (LDH) into the culture supernatants. LDH activity was quantified by the CytoTox 96 assay (Promega). Percentage lysis = [(OD of sample - OD of untreated cells)/(OD of 100% cell lysis - OD of untreated cells)]x100. Alternatively, cell death was quantified using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) according to the manufacturers' instructions. Briefly, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carbpxymethyoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium inner salt, was added to the cells after incubation for 6h with toxin. Release of a soluble formazan product, which requires dehydrogenase enzymes found only in living cells, was measured by the absorbance at 490 nm and this is directly proportional to the numbers of viable cells. Cell death shown represents decrease in viability compared with control (medium only), which is set at 0%. Percentage cell death= 100 - [OD of sample x 100/OD of untreated cells].

2.11 Detection of caspase-3

To determine if CyaA or CyaA derivatives induced apoptosis, the activation of caspase-3, a marker or apoptosis, was measured in cells following treatment with CyaA. J774 cells were treated for 6 h with CyaA or CyaA derivatives (0.1 – 10 μg/ml) in medium lacking FCS. Cells were washed and lysed in 300 μl lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 100 μM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin) and analyzed for protein concentration. 50 μl samples were added to 50 μl 2X reaction buffer (100 mM HEPES (pH 7.4), 150 mM NaCl, l0.2% CHAPS, 4 mM DTT) and 50 μM caspase-3 substrate (Ac-DEVD-aminofluorocoumarin, Alexis Corp.) and incubated at 37°C. Following the addition of the buffers and substrate, fluorescence caused by liberation of free aminofluorocoumarin was monitored over 60 min in a flourometer (excitation, 390 nm; emission, 510 nm), which corresponded to the breakdown of the substrate by caspase-3 and could therefore be used as a measure of caspase-3 activation.

2.12 Animals

Specific-pathogen free (SPF) female BALB/c, C3H/HeJ and C3H/HeN mice were purchased from Harlan UK. Ltd. (Bichester, England) and were housed in individually ventilated cages and were 6-10 weeks old at the initiation of experiments. The inbred BALB/c strain of mice is a standard laboratory stain used for immunological studies. C3H/HeJ mice carry a mutation in the *Tlr4* gene, resulting in a proline to histidine point-mutation at position 712, that renders these mice hypo-responsive to TLR-4 stimulation by LPS (Poltorak *et al.*, 1998). C3H/HeN mice are the corresponding wild-type mice and express a fully functional TLR-4 molecule. All experiments were performed according to regulations of the Irish Department of Health, The EU and the Ethics Committee of Trinity College Dublin.

2.13 Cell Lines

2.13.1 J774 macrophages

The J774A.1 murine macrophage cell line (J774), which was derived from BALB/c mice, was acquired from the European Collection of Cell Cultures (EACC) and was cultured in either cRPMI or cDMEM as required. Stocks were frozen in liquid nitrogen at passage 2 and 3 that were then thawed, grown and split when cells reached confluencey, usually after 4 days. J774 macrophages were used for experiments before passage 8.

2.13.2 Liquid nitrogen stocks

Stocks of murine cells lines ($4x10^6$/ml) were prepared in cRPMI or cDMEM supplemented with 50% FCS and 10% DMSO in a volume of 1 ml and placed in a cryotube (Nalge Nunc, Hereford, UK). This was frozen at -80°C in an insulated polystyrene container overnight before being stored in liquid nitrogen.

2.14 Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c, C3H/HeN and CeH/HeJ mice using a method modified from that described by Lutz *et al.* (Lutz *et al.*, 1999). Mice were euthanised and their femurs and tibiae removed and freed from the surrounding muscle tissue. The ball of the joint was removed from one end of the bone and the bone marrow was flushed out with RPMI-1640 medium using a 27G needle attached to a 10 ml syringe. Cell aggregates were then disintegrated by aspirating the cell mixture in a syringe with a larger needle to prevent damaging the cells. The resulting cells were pelleted by centrifugation at 1200 rpm for 5 minutes and resuspended in 1 ml of ammonium chloride lysis solution for 1 min to lyse red blood cells. Lysis was stopped by the addition of 9 ml of cRPMI and cells were centrifuged again and resuspended in 5 ml of fresh cRPMI. Cell viability was assessed using trypan blue. Immature BMDC were prepared by culturing cells at 1 x 10^6 cells/ml in a tissue culture flask in cRPMI supplemented with 40 ng/ml GM-CSF. GM-CSF was present in the form of supernatant from a GM-CSF-expressing J558 cell line (provided by

Nathalie Winter, Pasteur Institute, Paris, France). After 3 days in culture, an additional 15-20 ml of fresh cRPMI supplemented with GM-CSF was added to each flask of cells. On day 6 flasks were gently removed from the incubator and cell culture supernatants were removed carefully to eliminate contaminating non-adherent cells from the culture. 10 ml of warm sterile PBS was added to the flask and gently agitated before removing the cell suspension to a sterile 50 ml tube. 15 ml of sterile warmed EDTA (0.02%; Sigma, Poole, UK) was then added to each culture flask and cells were incubated for 10 mins at 37°C to loosen adherent cells. The loosened cells were removed with a pipette and added to the cells in PBS in the 50 ml tube and centrifuged at 1200 rpm for 5 mins. The resulting cell pellets were resuspended in 5 ml of cRPMI and cell viability and cell numbers was assessed by counting with trypan blue. Cells were then re-cultured at 1 x 10^6 cells/ml in cRPMI supplemented with 40 ng/ml GM-CSF. After a further 4 days of incubation, 15-20 ml of fresh cRPMI containing 40 ng/ml GM-CSF was added to each flask. 2 days later the loosely adherent cells were harvested, viability determined and the resulting immature DC were plated out at the required concentration (usually 1 x 10^6 cells/ml) in tissue culture plates and incubated overnight in complete RPMI with 10 ng/ml GM-CSF before treatment the following day.

2.15 Antigens, adjuvants and carrier molecules

2.15.1 Keyhole limpet hemocyanin (KLH)

Keyhole limpet hemocyanin (KLH) purified from the hemolymph of the keyhole limpet crab *Megathura crenulata* was purchased from Calbiochem (La Jolla, CA, USA). It was supplied at greater than 95% homogeneity and is certified to have less than 1 EU endotoxin/mg KLH.

2.15.2 NadA

NadA is a surface exposed antigen of *Neisseria meningitidis* expressed by all hyper-virulent disease causing strains (Bowe *et al.*, 2004). Recombinant NadA was received from Dr. Rino Rappuoli (Chiron, Siena, Italy) and was used as an antigen in nasal immunisation experiments. The NadA preparation contained 113 pg LPS / ug of protein.

2.15.3 Lipopolysaccharide (LPS)

LPS from *E. coli*, Serotype R515, was purchased from Alexis Biochemicals (Notthingham, UK). This product was certified to be 99.9% pure and to be a strong activator of TLR4 but free from contaminates that activate TLR2 or other TLRs. LPS, sometimes called endotoxin, is expressed in either μg/ml or in endotoxin units (EU)/ml where 1 EU = 100 pg LPS.

2.15.4 CpG-ODN

Phosphorothioate- stabilized oligodeoxynucleotide-containing CpG motifs (CpG-ODN) are ligands for TLR-9 and are potent immunomodulators. An effective sequence for the activation of murine immune responses was determined by Graham Donnelly in the laboratory, which was 5'-TCCATGACGTTCCTGATGCT-3' (CG motif underlined). A CpG-ODN molecule with this sequence was synthesised by Sigma Genosys (Cambridge, UK) and supplied as a dry powder. This was suspended in 1X TE buffer (10mM Tris-Cl pH 7.4, 1mM EDTA pH8) at 2 mg/ml and stored at -20°C before use in assays.

2.15.5 Chitosan

Chitosan is a polymer derived from the shells of crabs and shrimp and can be used as a carrier molecule for nasal vaccines (Baudner *et al.*, 2003, McNeela *et al.*, 2004). A commercial preparation of chitosan (Prototasan™) was purchased from Pronova Biomedial a.s., Oslo, Norway, and suspended in dH_2O. The LPS content was measured and found to be less than 1 pg/μg chitosan.

2.15.6 LTR72

LTR72 is a mutant of heat-labile enterotoxin (LT) of *E. coli* generated by site directed mutagenesis of the LT gene that encodes an alanine to arginine substitution at position 72 (Giuliani *et al.*, 1998). LTR72 has less than 1% of the enzymatic activity of wildtype LT and is less toxic. Recombinant LTR72 was received from Rino Rappuoli (Chiron,

Siena, Italy) and was used as an adjuvant in nasal immunisation experiments. The LRR72 preparation contained less than 25 pg LPS/mg of protein.

2.16 Immunization of mice

To investigate their adjuvant properties mice were immunised with antigen together with immunomodulatory molecules and the antigen specific immune response measured. Mice were immunised in either the hind foot-pads with a total of 50 μl (25 μl in each hind foot-pad), subcutaneously in the back with a volume of 200 μl or intranasally (i.n.) with a volume of 20 μl of vaccine formulations applied to the external nares of mice using a pipette. Mice were euthanised at the end of each experiment and the draining lymph nodes appropriate to the site of immunisation recovered and antigen specific immune responses assayed. The popliteal lymph nodes were recovered from mice immunised following foot-pad immunisation, the inguinal lymph nodes following subcutaneous immunisation in the back and the superior cervical lymph nodes following intranasal immunisation. Mice were boosted by additional immunisations with a repeat of the primary immunisation at the required time points. In the case of foot-pad immunisations, mice were boosted 21 days after the primary immunisation and sacrificed after a further 7 days. Mice immunised intranasally were boosted twice, 21 days and 40 days after primary immunisation, and sacrificed after a further 14 days.

2.17 Recovery of lymph nodes, blood and lungs

At the end of all immunisation experiments mice were sacrificed by cervical dislocation. Under sterile conditions, the thoracic cavity was opened and the heart was were cut with a scissors. Whole blood was then collected using a Pasteur pipette, typically 200-500 μl of blood was recovered from each mouse. The appropriate draining lymph nodes (indicated in Fig. 2.1) were then isolated and placed in cRMPI on ice before processing. The lungs of nasally immunised mice were removed and placed in RPMI supplemented with a protease inhibitor cocktail (Sigma, Dublin) and then stored at -20°C.

2.18 Preparation of serum for antibody ELISA

Whole blood collected from each individual mouse was allowed to clot at room temperature for 2 to 4 hours and then placed at 4°C for at least 2 hours. The clot was removed and the serum was centrifuged at 13,000 rpm in a desktop microfuge in a 1.5 ml flip top tube. The clear serum was aspirated off taking care not to disturb the pellet of red blood cells. The serum was stored at -20°C.

2.19 Assessment of antigen-specific T cell responses

Lymph node cell suspensions were prepared by pushing the isolated lymph nodes (in 3 ml of cRMPI) through a sterile cell–strainer in a sterile petri dish using the barrel of a 1 ml syringe. The cell-strainer was then washed with a further 3 ml of cRMPI and the 6 ml lymph node cell suspension was transferred to a 15 ml tube and centrifuged at 1200 rpm. The cell pellet was resuspended in 1 ml of cRPMI and the cells counted and viability checked with trypan blue (usually by adding 10 μl of cells to 40 μl of trypan blue and then counted using a haemacytometer). Lymph node cell suspensions were diluted to 1 x 10^6 cells/ml and stimulated with antigen, mitogens (positive control) or medium alone (negative control). Supernatants were removed after 72 hours for analysis of secreted IL-4, IL-5, IL-10 and IFN-γ concentrations. The concentration of IL-10 was measured using the a commercial ELISA kit (R&D Systems Inc., MN, USA) and the concentrations of IL-4, IL-5, and IFN-γ were measured by ELISA using antibodies described in table 2.2.

2.20 Detection of IL-4, IL-5 and IFN-γ

The concentrations of Il-4, IL-5 and IFN-γ in supernatants were measured by ELISA using antibody pairs (Pharmingen; Table 2.2). High binding certified ELISA plates (Greiner Bio-one) were coated with 50 μl/well of purified rat anti-mouse IL-5, IL-4 and IFN-γ (1 μg/ml) and incubated overnight at 4°C. The plates were washed 4 times with wash buffer (PBS/0.05% Tween 20) and incubated with 200 μl/well of blocking solution (PBS/10% milk powder (w/v)) for 2 hours at room temperature to prevent non-specific binding. Plates were washed 4 times with wash buffer and incubated overnight at 4°C with 50 μl/well of supernatant or serially diluted recombinant cytokine standard (Table 2.3). Plates were washed and then incubated for 2 hours at room temperature

with 50 µl/well of biotinylated anti-mouse IL-4, IL-5 or IFN-γ (1 µg/ml). Plates were washed once again, and were incubated for 40 minutes at room temperature with 50 µl/well of horse radish peroxidiase (HRP) conjugated-StrepAvidin diluted 1:2500 in PBS according to manufacturers instructions (PharMingen). Plates were washed and the necessary volume of o-phenylendiamine (OPD) substrate tablets (Sigma) dissolved in phosphate citrate buffer was prepared and 80 µl added to each well. Colouration was allowed to develop over 10-30 minutes and was stopped using 60 µl/well of 1 M H_2SO_4. Absorbance was read at 492 nm using a microtitre plate reader (Molecular Devices, VERSAmax tunable microplate reader). Cytokine concentrations in supernatants were determined with reference to the standard curve prepared from the recombinant cytokines of known concentration. The cells were cultured for a further 24 hours in fresh cRPMI and proliferation assayed.

2.21 Determination of T cell proliferation

Following stimulation with antigen and positive and negative controls, the proliferation of lymph node (1 x 10^6 cells/ml) cells was assayed. Lymph node cells were pulsed with 0.5 µCi of [^{3}H]-thymidine (Amersham Pharmacia Biotech, UK) per well (1:50 dilution [^{3}H]-thymidine in RPMI) and cultured at 37°C for a further 4-6 hours. The pulsed cells were either stored at -80°C or were harvested immediately onto glass fibre filter paper (Wallac) using an automatic cell harvester. The filters were dried and heat-sealed in plastic sample bags with 5 ml of non- aqueous scintillation fluid (BetaScint, Wallac). [^{3}H]-thymidine incorporation was measured using a Beta-plate scintillation counter (Wallac). Results are expressed as mean counts per minute (CPM) of [^{3}H]-thymidine incorporation for triplicate wells of spleen or lymph node cells.

2.22 Culture of T cell lines and clones and detection of cytokines

T cell lines and clones were established using a modification of methods described by Mills (Mills, 1999). Briefly, popliteal lymph node cells (1x$10^{6/}$ml) from immunised mice were cultured with KLH (50 µg/ml) for 5 days, after which, they were cultured with recombinant IL-2 (Hoffmann-La Roche Inc., NJ, USA; 5 units/ml) for 7 days. T cell lines and clones were then maintained in culture with syngenic γ-irradiated splenic

APC ($2x10^6$/ml) and KLH (50 μg/ml) for 4 to 5 days followed by culture with IL-2 (5 units/ml). After two rounds of antigen stimulation, T cell lines were cloned by limiting dilution. Cytokine production from T cells was tested at the end of the starve cycle, which is the day cultures were to be sublimated with IL-2.

2.23 Determination of serum IgG antibody and IgG isotype levels

The levels of antigen specific IgG antibodies in sera were determined by ELISA. KLH (5 μg/ml) or NadA (2 μg/ml) in PBS (50 μl/well), was added to flat bottomed medium-binding 96 well microtitre plates (Greiner Bio-One) and incubated overnight at 4°C. Plates were washed with wash buffer and blocked with 10% w/v dried milk powder in PBS (200 μl/well) for 2 hours at room temperature. Plates were washed and sera from immunised mice were serially diluted in PBS on the plates to determine the endpoint titres. After overnight incubation, the plates were washed and incubated with 50 μl/well of HRP-conjugated rat anti-mouse IgG monoclonal antibody (Zymed, San Franscisco, CA, USA) diluted 1:1000 acording to manufacturers instructions in PBS for 2 hours, biotin-conjugated IgG1 (0.5 μg/ml; PharMingen) or biotin-conjugated IgG2a (0.5 μg/ml; Pharmingen) in PBS for 1 hour. In the case of IgG1 and IgG2a, plates were then washed and incubated for 30 minutes at room temperature with 50 μl/well of HRP-conjugated streptavidin diluted 1:1000 in PBS according to manufacturers instructions. All plates were washed and then developed with 50 μl/well OPD in phosphate citrate buffer as described above. The enzymatic reaction was stopped with 50 μl/well 1 M H_2SO_4 and absorbance measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres determined by extrapolation of the straight part of the dilution curve to the OD_{492nm} value of the control serum for naïve mice.

2.24 Determination of lung IgA antibody levels

Lung that were removed from the thoracic cavity of naïve, immunised or infected mice after sacrifice and stored at -20°C in RPMI and protease inhibitor cocktail, were thawed on ice and homogenised. The levels of antigen-specific IgA antibodies in the lung was determined by ELISA. KLH (5 μg/ml) or NadA (2 μg/ml) in PBS was added to 96 well microtitre plates (50 μl/well)and incubated overnight at 4°C. Excess antigen was

removed and the plate washed with (PBS/ 0.05% Tween 20) and non-specific binding sites were blocked by incubating the plates with 10% w/v (PBS) dried powdered milk for 2 hours. Lung homogenates were serially diluted in PBS on the washed plates in a volume of 50 µl PBS to determine the endpoint titres using an initial dilution of 1:10. The plates were incubated overnight at 4°C. After washing, plates were incubated for 1 hour at room temperature with 50 µl/well of biotinylated anti-mouse IgA monoclonal antibody (Zymed, San Franscisco, CA, USA; 1:2500 in PBS). Plates were then washed and incubated for 30 minutes at room temperature with 50 µl/well of HRP-conjugated streptavidin diluted 1:1000 in PBS according to the manufacturers instructions. The plates were then washed and 80 µl of OPD (dissolved in phosphate citrate buffer) added and incubated at room temperature in the dark to allow the colour to develop. The enzymatic reaction was stopped by the addition of 60 µl/well 1 M H_2SO_4. Absorbance values were measured at 492 nm. Results are expressed as endpoint antibody titres determined by extrapolation to the OD_{492nm} value of the control samples.

2.25 Modulation of innate immune cell responses

To evaluate the responses of macrophages and immature DC to pathogen derived molecules, $1x10^6$ cells were cultured in tissue culture plates at 37°C with 5% CO_2, in either 96 or 24 well plates and allowed to rest and adhere over night. The following day, the medium on the plates was replaced with fresh warmed medium and immunomodulatory molecules were added at the appropriate concentrations. Polymyxin B was added to chelate LPS if required. In the case of immunomodulatory experiments with CyaA and CyaA derivatives, cells were pre-incubated with these proteins for two hours prior to the addition of TLR ligands. At the desired time points, supernatants were removed and tested for the presence of secreted cytokines and chemokines. Supernatants were typically stored at -20°C after collection. The evaluation of cell surface marker expression by FACS was performed on DC after 24 hour stimulation with immunomodulatory molecules.

2.26 Effect of blocking CD11b on the modulation of innate cell responses

J774 macrophages were cultured at $1x10^6$ cells/ml and CyaA was added at 1 μg/ml. Where required, purified anti-mouse CD11b antibody (M1/70) or purified rat IgG_{2b}, κ isotype control were added at a concentration of 10 μg/ml, 30 min prior to the addition of CyaA. Polymyxin B was added to the appropriate wells at a concentration of 10 μg/ml. 2 h after stimulation with CyaA, phosphorothioate-stabilised oligodeoxynucleotide-containing CpG motifs (CpG-ODN; 5'-TCCATGACGTTCCTGATGCT-3'; synthesized by Sigma-Genosys Ltd) was added at 10 μg/ml or LPS (Alexis Corp., UK) at 10 ng/ml. Supernatants were collected after 4 h and 24 h for analysis of secreted cytokine and chemokines by ELISA.

2.27 Detection of IL-10, IL-12, TNF-α and MIP-1α by immunoassay

Supernatants cell cultures were tested for the production of IL-10, IL-12, TNF-α or MIP-1α using the commercial Duo-Set ELISA kits (R&D Systems Inc., MN, USA) according to the manufacturers protocol with some modifications. Briefly, high-binding certified ELISA plates (Greiner Bio-one) were coated with 50 μl/well of the appropriate capture antibody in PBS (Table 2.2) and incubated at 4°C over night. Plates were then washed 4 times with wash buffer (PBS/0.05% Tween 20) and then incubated at room temperature with 200 μl/well of 5% sucrose, 1%BSA/PBS to block against non-specific binding. Plates were washed a further 4 times and the appropriate supplied recombinant standard was then serially diluted in 1%BSA/PBS in the appropriate wells of the plates with the required top working standard. The supernatants to be tested were then added to the plates and diluted 1 in 2 with 1% BSA/PBS (25 μl of supernatants with 25 μl of 1% BSA/PBS). Plates were incubated at 4°C overnight and then washed four times in wash buffer before the addition of 50 μl/well of the appropriate secondary biotinylated antibody diluted in 1%BSA/PBS. After incubation for 2 hours at room temperature, plates were washed 4 times in wash buffer. Streptavidin conjugated HRP diluted 1 in 200 with 1%BSA/PBS (50 μl) was then added to each well and plates were then incubated in the dark for 20 minutes at room temperature. Plates were washed 4 times in wash buffer and colour change was developed by the addition of the commercial 80 μl/well TMB substrate (Sigma, Poole, UK). The colour was allowed to develop over

10-30 minutes and was stopped using 60 µl/well of 1 M H_2SO_4. Absorbance was read at a wavelength of 450 nm using a microtitre plate reader. Cytokine concentrations in supernatants were determined by reference to the standard curve of recombinant cytokine of known concentration.

2.28 Detection of IL-6 by immunoassay

The concentrations of IL-6 in supernatants removed from DC or macrophages were determined by ELISA using the paired antibodies and recombinant standard listed in tables 2.2 and 2.3. Briefly, high binding certified ELISA plates (Greiner Bio-one) were coated with 50 µl/well of purified rat anti-mouse IL-6 (BD Pharmingen; 1 µg/ml) and incubated overnight at 4°C. The plates were washed 4 times with wash buffer (PBS/0.05% Tween 20) and incubated with 200 µl/well of blocking solution (PBS/10% milk powder (w/v)) for 2 hours to prevent non-specific binding. Plates were washed 4 times with wash buffer and incubated overnight at 4°C with 50 µl/well of supernatant or serially diluted recombinant cytokine standard (Table 2.3) with a top working standard of 5000 pg/ml. Plates were washed and then incubated for 2 hours at room temperature with 50 µl/well of biotinylated rat anti-mouse IL-6 (BD Pharmingen; 1 µg/ml). Plates were washed once again, and incubating for 40 minutes at room temperature with 50 µl/well of horseradish peroxidase (HRP) conjugated-StrepAvidin (BD PharMingen) diluted 1 in 2500 according to manufacturers instructions in PBS. Plates were washed and the necessary volume of o-phenylendiamine (OPD) substrate tablets (Sigma) dissolved in phosphate citrate buffer was prepared and 80 µl added to each well. Colouration was allowed to develop over 10-30 minutes at room temperature in the dark and the reaction stopped using 60 µl/well of 1 M H_2SO_4. Absorbance was read at 492 nm using a microtitre plate reader (Molecular Devices, VERSAmax tunable microplate reader). Cytokine concentrations in supernatants were determined with reference to the standard curve prepared from the recombinant IL-6 of known concentration.

2.29 Flow Cytometry Analysis

The expression of surface markers on DC was determined by flow cytometery analysis using a FACS Caliber™ flow cytometer (Becton-Dickinson, San Jose, CA) following staining with fluorescently labelled antibodies specific for different cell surface markers. DC to be analysed were cultured in 24 well plates at $1x10^6$ cells/well in 1 ml of medium, with a total of $3x10^6$ cells to be analysed per treatment group. Supernatants were removed from the DC and replaced with 1 ml of PBA per well, and cells removed from the surface of the wells with a cell scraper or the barrel of a 1 ml syringe and kept on ice unless required. Cells for each treatment were centrifuged in a FACS tube at 1200 rpm for 5 minutes, the supernatant discarded and the cells washed in 4 ml of PBA. Cells were centrifuged, the supernatant discarded and cells were then incubated in 1 ml of 50% FCS/50% PBA for 20 minutes at room temperature. A further 3 ml of PBA was added to each tube and cells centrifuged. The supernatants were discarded and resuspended in 100 μl of PBA for each antibody cocktail. Typically, each group of cells was tested for the expression of three surface markers with three different antibodies conjugated to FITC, PE and PerCP florophors that can be distinguished by the flow cytometer. Cells were incubated with antibodies and the appropriate isotype controls at the required concentrations (Table 2.4) for 20 minutes protected from the light. In the case of the PerCP florophore, the primary antibodies were supplied conjugated to biotin, and following staining with these antibodies cells were then incubated with streptavidin conjugated PerCP following a 20 minute incubation and a wash step in 4 ml PBA. All cells were finally washed in 4 ml PBA and resuspended in 200 μl PBA and 50,000 cells read per group on the flow cytometer. The results were analysed using CELLQuest™ software (BD Biosciences, CA, USA).

2.30 Detection of intracellular cytokines by FACS analysis

A minimum of $2x10^6$ cells cultured in cRPMI in a 24 well plate were needed analyse each set of cytokines. Cells were stimulated for 2 h with the Con A (5 μg/ml; Sigma, Dublin), PMA (10 ng/ml; Sigma, Dublin) and ionomysin (1 μg/ml; Sigma, Dublin) and cultured for a further 4 h with Brefeldin A (10 μg/ml). Cells were transferred to 1.5 ml tubes and washed well with 0.5 ml PBA and pelleted by centrifigateion at 900 g for 5 minutes. The supernatant was discarded and the cells were resuspended in 100 μl

50%FCS/PBA and incubated at room temperature for 20 minutes. Cells were washed 3 times with 0.5 ml PBA and resuspended in a final volume of 100 μl PBA. An antibody against the surface expressed CD3 (Table 2.4) was added and incubated on ice for 45 minutes in the dark. Cells were washed and streptavidin conjugated PercP was then added. Cells were then incubated in the dark for 20 minutes. Cells were washed 3 times before being resuspended in 50 μl of Fixation Buffer A from the commercial Fix and Perm kit (Caltag, CA, USA) mixing very gently to resuspended. Cells were incubated at room temperature for 15 minutes and were then washed 3 times in PBA and resuspended in 50 μl Permeabilisation Buffer B from the Fix and Perm kit together with antibodies against the target cytokines for 15 minutes in the dark at room temperature. Cells were then washed 3 times in PBA and resuspended in 100 μl PBA and analysed immediately using a FACS Caliber™ flow cytometer.

2.31 Morphological assessment of CyaA treated J774 cells

J774 cells were plated on glass coverslips one day before treatment. Cells were treated with 1-10 μg/ml of CyaA or NA-CyaA, 1 μM cyclohexamide or medium only for 6 h. After treatment, coverslips were removed and cells were fixed with 3% paraformaldehyde in PBS, washed and mounted on glass slides for analysis. Cells were visualised under white light using a BX51 microscope (Olympus) with attached camera and images acquired using AnalySIS® software.

2.32 Statistical Analysis

Statistical analysis was performed using the computer based mathematical package, InStat (GraphPad Software, San Diego, CA, USA). Statistical differences in mean antibody or cytokine concentrations were determined by Analysis of Variance (ANOVA).

2.33 Transcriptomic analysis of immunomodulation by CyaA

2.33.1 Treatment of cells

J774 macrophages were grown as described above and plated at $1x10^6$ cells/ml overnight in 24 well plates. Cells were treated with CyaA (1 μg/ml), CpG (10 μg/ml), CyaA and CpG or untreated (medium alone). At least 3 biological replicates of $1x10^6$ cells were stimulated with each treatment for each time point examined. The RNA from the biological replicates was pooled for each group and time point.

2.33.2 Isolation of total RNA

Total RNA was isolated from J774 macrophages by combining and modifying a Tri-reagent protocol developed in the laboratory by Dr. Michelle Armstrong and the commercial RNeasy Kit protocol (Qiagen, UK).

The supernatants were removed from J774 macrophages stimulated with immunomodulatory molecules for the appropriate amounts of time and stored for analysis by ELISA. All work was performed on ice, and centrifugation performed at 4°C, except where otherwise stated. 1 ml of commercial Tri-reagent (Sigma) was added to each well containing $1x10^6$ cells and the cells were scraped from the bottom of the well using the barrel of a 1 ml syringe. These samples could be processed immediately, although they were usually stored at -80°C prior to processing, and were stable for up to one month in Tri-reagent. Samples were thawed (if necessary), mixed well for one minute and allowed to stand at room temperature for 5 minutes. Chloroform (200 μl; Sigma) was added to each 1 ml sample and mixed well for 15 seconds. Samples were centrifuged in a desktop microfuge at 13,000 rpm for 15 minutes, during which time the samples separated into three distinct phases. The top aqueous phase, containing the RNA, was removed to a fresh tube taking care not to disturb the lower layers containing DNA, protein, carbohydrate and other cellular components. 500 μl of isopropanol (Sigma) was added to each tube and mixed well and allowed to stand at room temperature for 10 minutes. The RNA was pelleted by centrifugation at 13,000 rpm in a desktop microfuge and the supernatant removed taking care not to disturb the white coloured pellet of RNA on the side of the tube. 1 ml of 75% EtOH was added to each

pellet and it was frozen over night at -80°C. In this state, the RNA is stable for up to one year. Tubes were centrifuges for 15 minutes and the supernatant carefully removed. The RNA pelleted would be seen as a small white spot at the end of the tube. The pellet was allowed to air dry at room temperature for 5 minutes. The RNA from each biological replicate (containing RNA from $1x10^6$ cells) was pooled in a total of 100 µl for each treatment.

The total RNA was then further purified using the Qiagen RNeasy kit and supplied buffers at room temperature, unless otherwise stated. 350 µl of RLT buffer, which had been supplemented with 14.3M β-mercaptoethanol, was added to each sample and mixed well. EtOH (100%; 250 µl) was then added to each sample, mixed well and applied to an RNeasy Spin Column in a collection tube, which was centrifuged in a microfuge at 10000 rpm for 15 seconds. The flow through and collection tubes were discarded and the column placed in a fresh 2 ml collection tube. 500 µl RPE buffer, which had been diluted with EtOH according to the manufacturers instructions, was added to each sample. Each sample was centrifuged at 10000 rpm for 2 minutes and the flow through discarded. The column was centrifuged, with the lid open, for a further 1 minute to fully dry the membrane. Each column was placed in a fresh 1.5 ml collection tube and 50 µl of RNase free dH_2O applied directly to the membrane. This was centrifuged in a microfuge for 1 minute at 10000 rpm. The 50 µl elute was reapplied to the membrane and centrifuge again for 1 minute to improve the yield. Pure total RNA was stored at -80°C.

2.33.3 Quantification and quality assessment of RNA by spectrophotometry

After purification of RNA was complete, an aliquot of each RNA sample was taken before freezing the sample to assess RNA quality and concentration. Usually, a 1 in 50 dilution of total RNA was made in RNase free water in a volume of 150 µl. This was read in a spectrophotometer at a wavelength of 260 nm.

RNA concentration was determined from the following formula;

[OD260 x dilution factor x 0.04] = total RNA concentration (µg/µl)

Quality and purity of each RNA sample was determined by then measuring each sample at a wavelength of 280 nm. The ratio of OD260 to OD280 was calculated, [OD260/OD280]. A ratio of between 1.8 and 2.0 indicated good quality pure RNA.

2.33.4 Quantification and quality assessment of RNA using a Bioanalyzer

To confirm the stability and concentration of the purified total RNA, 100 ng of total RNA (100 ng/μl) was analysed on a nano-RNA chip using a Bioanalyzer machine and the supplied commercial reagents (Agilent Technologies, Germany). To do this, the electrode probes of the Bioanalyser were first cleaned using RNAzap (Sigma) and RNase free dH_2O as described in the manufacturers instructions. Nano gel matrix was prepared by filtering a 65 μl aliquot on the supplied spin filter column at 1500g (4000 rpm in a desk top microfuge) for 10 minutes. The gel-dye mix was prepared by adding 1 μl Nano dye to the 65 μl filtered Nano-gel matrix, this was stored in the dark until used. 9 μl of the gel dye mix was added to the appropriate wells of a fresh Nano 6000 RNA chip, which was then primed for 30 seconds in the chip priming station. Gel dye mix (9 μl/well) was added to the wells as indicated in the manufacturers instructions, while 5 μl of the RNA 6000 Nano Marker was added to the sample and ladder wells. Each RNA sample and the supplied RNA ladder were first denatured by heating at 70°C for 2 minutes before 1 μl of each was added to the appropriate wells. The chip was vortexed for one minute at 2400 rpm in the supplied vortex before being placed in the Bioanalyzer machine and samples tested using the pre-programmed RNA Nano program. The 18S and 28S ribosomal RNA peaks were identified in each sample by reference to the spiked in RNA 6000 Marker added to each sample. Good quality RNA samples were identified by calculating the 28S/18S peak ratio, samples with a ratio of between 1.6 and 2.0 were deemed acceptable. In addition, the traces for each sample were examined for evidence of RNA degradation.

2.33.5 First Strand cDNA synthesis.

The One-cycle cDNA Systhesis kit purchased from Affymetrix (High Wycombe, UK) was used. First, the RNA concentration in each sample was re-calculated based on the Bioanalyser results. The supplied Poly-A controls were diluted according to the

manufacturers instructions based on the total amount of total amount of RNA from each experimental sample to be tested. Total RNA (3 μg) from each experimental sample was used as the starting template, to which was added 2 μl of the diluted Poly-A controls, 2 μl of the T7 oligo primer (50 μM) and was brought to a final volume of 12 μl with RNase free water in a 0.2 μl PCR tube. This was incubated at 70°C for 10 minutes and then 4°C for at least 2 minutes. After which, 4 μl of 5X 1st Strand Reaction Mix, 2 μl DTT (0.1M) and 1 μl dNTP (10 mM) was added to each sample, bringing the total volume in each sample to 19 μl. This was incubated at 42°C for 2 minutes after which, 1 μl of Superscript II was added to each sample bring it to a total volume of 20 μl. This was incubated at 42°C for 1 hour, followed by incubation at 4°C for at least 2 minutes.

2.33.6 Second Strand cDNA Synthesis

The following were added to each sample, 91 μl RNase free water, 30 μl 5X 2nd Strand Buffer, 3 μl dNTP (10 mM), 1 μl *E. coli* DNA ligase, 4 μl *E. coli* DNA polymerase I and 1 μl RNase H. This brought the total volume of each sample to 150 μl. Samples were mixed and transferred to a 0.5 ml tube and incubated at 16°C for 2 hours. After this, 2 μl of T4 DNA polymerase was added to each sample and were incubated for a further 5 minutes at 16°C before the addition of 10 μl EDTA (0.5 M). Samples were not allowed to cool on ice for an extended period and were instead cleaned up immediately, as advised by Maureen Padden (Conway Institute, UCD, Dublin).

2.33.7 Clean-up of double stranded cDNA

The Sample Clean-up Module purchased from Affymetrix was used in the following steps. All supplied buffers were prepared according to the manufacturers instructions. Each cDNA synthesis reaction was transferred to a fresh 1.5 ml tube, followed by the addition of 600 μl cDNA binding buffer. Samples were vortexed and a yellow colour observed in each sample. Each sample (500 μl) was transferred into a cDNA Clean-up Column, and centrifuged in a microfuge for 1 minute at 10,000 rpm. The flow through was discarded from the attached collection tube and the remaining amount of each sample was applied to the column and centrifuged again. The column, on which the

cDNA was now bound, was transferred to a fresh collection tube and 750 µl cDNA Wash Buffer was added to each. This was centrifuged at 10,000 rpm for 1 minute and the flow through discarded. The columns were then centrifuged for 5 minutes at 10,000 rpm with the caps open to dry the column membrane. The column was then placed in a 1.5 ml collection tube and 14 µl Elution Buffer was applied directly to each column membrane. This was incubated at room temperature for 1 minute, before centrifugation at 13,000 rpm to collect the cDNA. Approximately 12 µl was recovered from each column.

2.33.8 In vitro transcription labelling reaction

The Genechip IVT Labeling Kit purchased from Affymetrix (UK) was used to prepare biotin labelled cRNA. The following were added to the 12 µl of cDNA recovered following the clean up step, 8 µl RNase free water, 4 µl 10X IVT labelling buffer, 12 µl IVT NTP mix and 4 µ IVT enzyme mix. This brings each sample to a volume of 40 µl. 2 µl (1 µg) of the provided 3'-labeling control was used as a positive control template for the IVT reaction, and this was brought to the correct volume with an additional 10 µl of RNase free water. The samples were incubated for 16 hours at 37°C, and can be stored at -80°C, but were in fact purified immediately in all experiments.

2.33.9 Clean-up of biotin labelled cRNA from IVT reaction

The Sample Clean-up Module (Affymetrix, UK) was used in this step, all reagents were prepared according to manufacturers instructions. RNase free water (60 µl) was added to each sample and was vortexed and moved to a fresh 1.5 ml tube. IVT Binding Buffer (350 µl)was added to each sample and was mixed by vortexing after which 250 µl 100% EtOH was added, and pippetted up and down to mix. All 700 µl of each sample was applied to an IVT RNA Clean-up Spin Column, which was centrifuged for 15 seconds at 10,000 rpm in a desktop microfuge. The column was placed in a fresh collection tube and 500 µl of IVT Wash Buffer was added to each before centrifuging again for 15 seconds at 10000 rpm. The flow through was discarded and 500 µl 80% EtOH added to each column. Each column was centrifuged at 10,000 rpm for 15 seconds and the flow through discarded. Columns were then centrifuged at 13,000 rpm

for 5 minutes with the caps open to dry the column membrane thoroughly. The columns were moved to a fresh 1.5 ml tube and 11 μl RNase free water added directly onto the column membrane. This was then centrifuged at 13,000 rpm for 1 minute. A further 11 μl RNase free water was added to the membrane and centrifuged at 13,000 rpm with the cap open. This can be stored at -80°C if not immediately quantified and fragmented.

2.33.10 Quantification of cRNA.

Biotin labelled cRNA was quantified by spectrophotometry as described above. A 1 in 100 dilution of each sample was made in RNase free water, $OD_{260\ nm}$ and $OD_{280\ nm}$ measured and the amount of cRNA present calculated. The yield was recalculated, according to the instructions provided by the manufacturers, to adjust for any potential carry over of total RNA in the samples. To assess the quality and stability of the cRNA, 1 μl of each sample was retained and diluted to 400 ng/μl in RNase free water and analysed on the Bioanalyser, as described above.

2.33.11 Fragmentation of cRNA

Using the adjusted yield, 20 μg of cRNA was fragmented using the Sample Clean-up Module (Affymetrix, UK). 8 μl of 5X Fragmentation buffer was added to 20 μg cRNA and each sample brought to a final volume of 40 μl with RNase free water. This was incubated for 35 minutes at 95°C followed by incubation at 4°C for 10 minutes. Samples were placed on ice for a further 10 minutes before being stored at -80°C. The final concentration of cRNA is 0.5 μg/μl, 1 μl was removed and diluted to 150 ng/μl for analysis on the Bioanalyser to confirm successful fragmentation prior to hybridisation to GeneChip microarrays.

2.33.12 Hybridization of fragmented biotin labelled cRNA to GeneChip

Each sample of fragmented cRNA was hybridised to a Mouse 430 2.0 GeneChip (Affymetrix, UK) according to manufacturers instructions by Alison Murphy (Transcriptomics Core Facility, Conway Institute, UCD). Each chip was read in an Affymetrix Microarray Scanner and the data read using the MAS v5.0 software (Affymetrix, UK).

2.33.13 Analysis of Microarray data.

Raw data, in the form of Chip Files (.chp), were returned by the Transcriptomics Core Facility (Conway Institue, UCD, Dublin). These were analysed using Genespring v7.2 software (Aligent Silicon Genetics). Normalisation of data from each chip was performed, first by applying an algorithm to normalise the data across all the chips to the 50^{th} percentile and then an algorithm to normalise each gene within the chip to the median in each independent experiment. Initially, a list was prepared of all the genes that were present or marginally up- or down-regulated across all chips in the experiment, and from this list all other lists were extracted. A cut off of 3-fold expression, of either up or down regulation, was used in the preparation of all gene expression lists.

Table 2.1 PCR reactions

Template	Primer 1	Primer 2	Anealing temp.	Extention Time	Cycles	Product size
B pertussis genomic DNA	PAB5	PAB6	63-68°C	6 minutes	35	2594 bases
B pertussis genomic DNA	PAB7	PAB2	52-57°C	6 minutes	35	2536 bases
B pertussis genomic DNA	PAB3	PAB4	44-46°C	2 minutes	35	575 bases
pAPB9	pUC Fwd	PAB27	60°C	6 minutes	35	400 bases
pAPB9	PAB17	PAB29	46-54°C	4 minutes	35	1700 bases

Table 2.2 ELISA Antibodies

Specificity	Use	Clone	Conjugated	Source (Prod Code)	Stock Conc.	Working Conc.
IL-4	**ELISA Capture**	**11B11**	**No**	**BD Pharmingen (554434)**	**0.5 mg/ml**	**1 µg/ml**
IL-4	**ELISA Detection**	**BVD6-24G2**	**Biotin**	**BD Pharmingen (554393)**	**0.5 Mg/ml**	**1 µg/ml**
IL-5	**ELISA Capture**	**TRFK5**	**No**	**BD Pharmingen (554393)**	**0.5 mg/ml**	**1 µg/ml**
IL-5	**ELISA Detection**	**TRFK4**	**Biotin**	**BD Pharmingen (554397)**	**0.5 mg/ml**	**1 µg/ml**
IFN-γ	**ELISA Capture**	**R4-A26**	**No**	**BD Pharmingen (551216)**	**1 mg/ml**	**1 µg/ml**
IFN-γ	**ELISA Detection**	**XMG1.2**	**Biotin**	**BD Pharmingen (554410)**	**0.5 mg/ml**	**1 µg/ml**
IL-6	**ELISA Capture**	**MP5-20FP**	**No**	**BD Pharmingen (554400)**	**0.5 mg/ml**	**1 µg/ml**
IL-6	**ELISA Detection**	**MP5-32C11**	**Biotin**	**BD Pharmingen (554402)**	**0.5 mg/ml**	**1 µg/ml**
IgG	**ELISA Detection**	**LO-MG-7, LO-MG1-2, LO-MG3-7**	**HRP**	**Zymed**	**N/A**	**1:1000 dilution**
IgG1	**ELISA Detection**	**A85-1**	**Biotin**	**BD Pharmingen (553441)**	**0.5 mg/ml**	**0.05 µg/ml**
IgG2a	**ELISA Detection**	**R19-15**	**Biotin**	**BD Pharmingen (553388)**	**0.5 mg/ml**	**1 µg/ml**
IgA	**ELISA Detection**	**62-6720**	**HRP**	**Zymed**	**N/A**	**1:2500**
IL-10	**ELISA Capture**	**N/A**	**No**	**R&D Systems (DY-417E)**	**360 µg/ml**	**2 µg/ml**
IL-10	**ELISA Detection**	**N/A**	**Biotin**	**R&D Systems (DY417E)**	**72 µg/ml**	**400 ng/ml**
IL-12p70	**ELISA Capture**	**N/A**	**No**	**R&D Systems (DY419)**	**720 µg/ml**	**4 µg/ml**

IL-12p70	**ELISA Detection**	**N/A**	**Biotin**	**R&D Systems (DY419)**	**72 µg/ml**	**400 ng/ml**
TNF-α	**ELISA Capture**	**N/A**	**No**	**R&D Systems (DY410)**	**114 µg/ml**	**0.8 µg/ml**
TNF-α	**ELISA Detection**	**N/A**	**Biotin**	**R&D Systems (DY410)**	**27 µg/ml**	**150 ng/ml**
MIP-1α	**ELISA Capture**	**N/A**	**No**	**R&D Systems (DY450)**	**72 µg/ml**	**0.4 µg/ml**
MIP-1α	**ELISA Detection**	**N/A**	**Biotin**	**R&D Systems (DY450)**	**18 µg/ml**	**100 ng/ml**

Table 2.3 Recombinant cytokine and chemokine standards

Molecule	Source (Product Code)	Stock Concentration	Top Working Standard
IL-4	**BD Pharmingen (550067)**	**25 ng/ml**	**2500 pg/ml**
IL-5	**BD Pharmingen (554581)**	**200 ng/ml**	**2500 pg/ml**
IFN-γ	**BD Pharmingen (554587)**	**1 μg/ml**	**20 ng/ml**
IL-6	**BD Pharmingen (554582)**	**5μg/ml**	**5000 pg/ml**
IL-10	**R&D Systems (DY417E)**	**70 ng/ml**	**2000 pg/ml**
IL-12p70	**R&D Systems (DY419)**	**60 ng/ml**	**2500 pg/ml**
TNF-α	**R&D Systems (DY410)**	**270 ng/ml**	**2000 pg/ml**
MIP-1α	**R&D Systems (DY450)**	**90 ng/ml**	**500 pg/ml**

Table 2.4 FACS Antibodies

Specificity	Clone	Conjugated	Source (Product Code)	Stock Conc.	Working Conc.
CD80	**16-10A1**	**PE**	**BD Pharmingen (553769)**	**0.2 mg/ml**	**7 ng/ml**
CD86	**GL1**	**FITC**	**BD Pharmingen (553691)**	**0.5 mg/ml**	**15 ng/ml**
CD40	**HM40-3**	**FITC**	**BD Pharmingen (553723)**	**0.5 mg/ml**	**15 ng/ml**
ICAM-I	**3E2**	**PE**	**BD Pharmingen (553253)**	**0.2 mg/ml**	**7 ng/ml**
MHC-II	**M5/114.15.2**	**PE**	**BD Pharmingen (557000)**	**0.2 mg/ml**	**1.5 ng/ml**
CD11c	**HL3**	**Biotin**	**BD Pharmingen (553800)**	**0.5 mg/ml**	**15 ng/ml**
Streptavidin	**N/A**	**PerCP**	**BD Pharmingen (554064)**	**0.2 mg/ml**	**4 ng/ml**
CD3	**1452C11**	**Biotin**	**BD Pharmingen (553060)**	**0.5 mg/ml**	**10 µg/ml**
IL-4	**N/A**	**FITC**	**Caltag (RM9051)**	**100 µg/ml**	**500 ng/ml**
IL-10	**N/A**	**PE**	**Caltag (RM9104)**	**100 µg/ml**	**500 ng/ml**

Table 2.5 Oligonucleotides used in construction of expression plasmids				
Primer name	Primer sequence	Underlined restriction site	Anneals to plasmid	Anneals to plasmid at base number
PAB5	5'-CGCCGGTACCATGCAGCAATCGCATCAGGCT-3'	*Kpn*I	pAPB4	22
PAB6	5'-TGGTGAATTCGCTCTTGCCCG-3'	*Eco*RI	pAPB4	2574
PAB7	5'-AAGAGCGAATTCACCACATTCGTCG-3'	*Eco*RI	pAPB5	18
PAB2	5'-CGCGGATCCTCAGCGCCAGTTGACAGCCA-3'	*Bam*HI	pAPB5	2518
PAB3	5'-CGCGGATCCGAGGGCATGTCATGCTTCCGTCCGCC-3'	*Bam*HI	pAPB1	31
PAB4	5'-CGCGGCGAAGCTTTCAGGCGGTGCCCCGGC-3'	*Hind*III	pAPB1	558
pUC Forward	5'-CCCAGTCACGACGTTGTAAAACG	N/A	pAPB9	1
PAB27	5'-CAACCCCCAATCGGA*TCC*C*GC*GGCG*GC*CACGCCCAATCCTTTG-3'	*Bam*HI	pAPB9	281
PAB17	5'-CAAAGGATTGGGCGTG*GC*CGCC*GC*G*GGA*TCCGATTGGGGGTTG-3	*Bam*HI	pAPB9	323
PAB29	5'-CGTAGATCTCCATGGGACTGAGC-3'	*Nco*I	pAPB9	1961

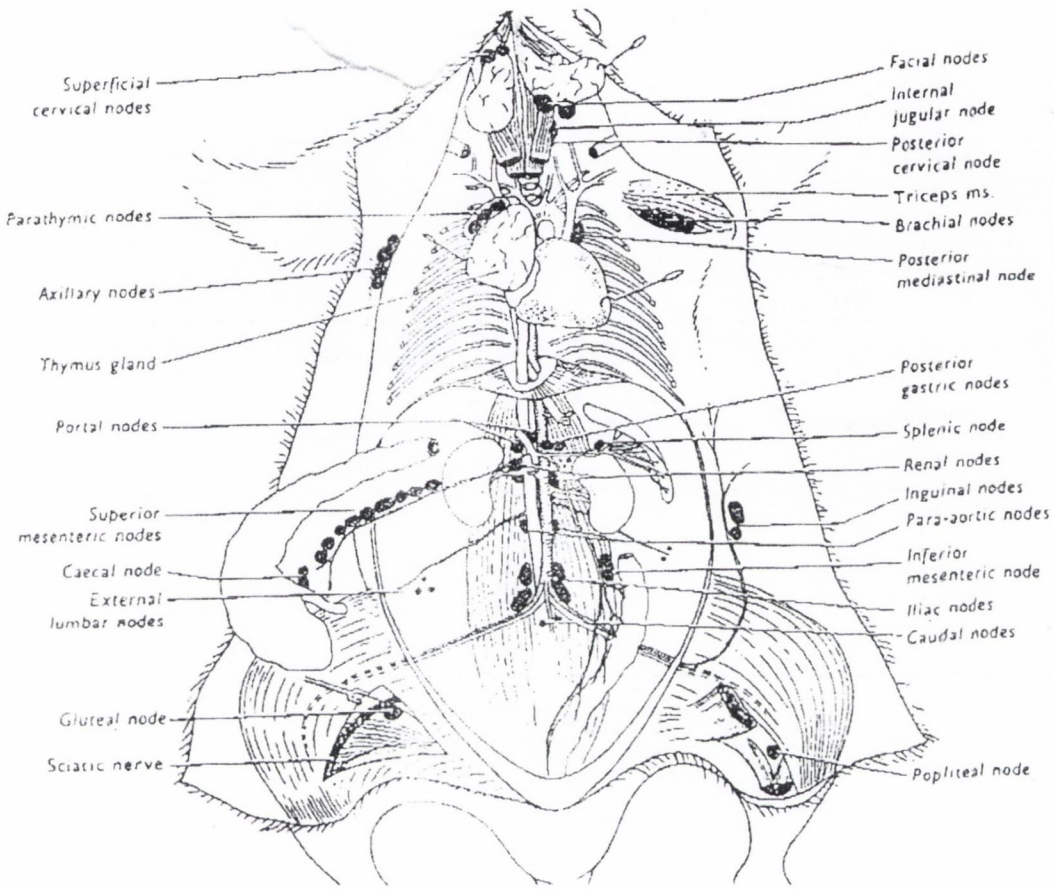


Figure 2.1 Position of lymph node organs in the mouse.

Lymph nodes are important organs that produce lymph, the substance that baths the body's organs and muscles. Local lymph nodes play an important role in the immune system where the cells of the innate and adaptive immune system interact.

Chapter 3

Cloning, expression, purification and characterisation of CyaA and CyaA derivatives

3.1 Introduction

Adenylate cyclase toxin (CyaA), a secreted virulence factor of *Bordetella pertussis,* interacts with cells of the innate immune system and acts as an adjuvant, enhancing antigen-specific antibody titres against a co-injected protein (Hormozi *et al.*, 1999). CyaA is post-translationally acylated at Lys^{983} and has two major domains (Basar *et al.*, 2001, Sakamoto *et al.*, 1992). The C-terminal haemolytic domain interacts with cell membranes forming pores (Gray *et al.*, 1998). It has been reported that the post-translational acylation of Lys^{983} of CyaA by the specific accessory protein CyaC is necessary for CyaA to interact with target cells and for CyaA to form pores in target cell membranes (Heveker *et al.*, 1994). The enzymatic domain of CyaA, located in the N-terminal 400 amino acids, becomes fully activated when it binds eukaryotic calmodulin upon its entry into the target cell cytosol (Wolff *et al.*, 1980). This enzymatic activity catalyses increased production of intracellular cAMP, which has been shown to perturb immune cell functions (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987). CyaA binds to cells of the innate immune system expressing the $\alpha_M\beta_2$ integrin CD11b/CD18 through the 1166 to 1281 amino acid region at the C-terminal of the protein (El-Azami-El-Idrissi *et al.*, 2003). Treatment of human DC with a commercial preparation of CyaA was shown to stimulate the upregulation of CD80, CD86 and CD83, which are surface molecules associated with DC activation (Bagley *et al.*, 2002b). CyaA was also shown to increase antigen-specific antibody production when co-injected with antigen in mice (Hormozi *et al.*, 1999, Macdonald-Fyall *et al.*, 2004). CyaA has been shown to have an important role in modulating the immune response during *B. pertussis* infection, however, its mechanism of action is not clearly understood. The aim of these studies was to determine how CyaA modulates both the innate and adaptive immune system and to examine if CyaA has potential as an anti-inflammatory molecule.

A pure preparation of CyaA is essential to properly investigate its immunomodulatory properties. Contamination of protein preparations with molecules that are themselves immunomodulatory may mask the immune response elicited by a recombinant protein preparation. Contamination with even low concentrations of immunomodulatory molecules, that would in itself not generate a response in immune

cells, may act in synergy with the molecule under study to alter the immune response generated. To produce sufficient material of high purity a His-tagged CyaA was produced in *E. coli* and purified for use in immunomodulatory experiments. CyaA had been shown to modulate the activity of human monocyte derived DC, this modulation was shown to be mediated by the supraphysiological levels of intracellular cAMP catalysed by CyaA (Bagley *et al.*, 2002b). A role for the enzymatic activity of CyaA in the virulence of *B. pertussis* has also been demonstrated (Ehrmann *et al.*, 1992, Gross *et al.*, 1992) and in the inhibition of antibacterial oxidative responses (Pearson *et al.*, 1987). These results demonstrate that the enzymatic activity of CyaA to catylyse the formation of cAMP plays a role in its ability to modulate aspects of the immune response. However, enzymatically inactive toxoid derivatives of CyaA used to deliver T cell epitopes and antigens demonstrate an ability to stimulate an immune response similar to that of similar enzymatically active CyaA molecules that retain the ability to increase cAMP. (Dadaglio *et al.*, 2003, Fayolle *et al.*, 1999). In order to fully understand the role of the enzymatic activity of CyaA in immunomodulation a mutant of CyaA was generated by site-directed mutagenesis of the *cyaA* gene that ablated enzymatic activity. This inactive adenylate cyclase derivate of CyaA (iAC-CyaA) was expressed and purified in *E. coli* for immunomodulatory experiments.

Acylation of CyaA plays an important role in the virulence of *B. pertussis* (Gross *et al.*, 1992) and is necessary for interaction with target cells and to cause lysis (Barry *et al.*, 1991, Rogel *et al.*, 1989). However, non-acylated CyaA (NA-CyaA) can bind CD11b expressing cells (El-Azami-El-Idrissi *et al.*, 2003) and T cells (Hewlett *et al.*, 1993). Although no increase in the concentrations of intracellular cAMP was detected, it was speculated that the lack of acylation might result in a defect in membrane interaction or the translocation of the N-terminal domain of CyaA across the eukaryotic cell membrane (Hewlett *et al.*, 1993). However, it has been shown that cAMP induction by CyaA occurs independently of lysis (Gray *et al.*, 1998) but that the presence of the N-terminal (Gray *et al.*, 2001), although not the ability to catalyse cAMP formation (Ehrmann *et al.*, 1992), reduces the lytic activity of CyaA. In order to better understand the role of acylation of CyaA in the activity of CyaA a non-acylated derivative (NA-CyaA) was produced by expressing the *cyaA* gene in the absence of the

cyaC gene in *E.coli*. This NA-CyaA was purified from *E. coli* and used to investigate the role of acylation in cell lysis and immunomodulation by CyaA.

Although a commercial preparation of recombinant acylated adenlyate cyclase toxin is available, it is important to be able to directly compare the effects of the CyaA protein to those of the derivatives constructed to examine the roles of the individual domains of CyaA. For this reason, all CyaA proteins were expressed in the same bacterial strain from the same expression vector and purified according to the same protocols.

3.2 Results

3.2.1 Plasmid construction.

3.2.1.1 His-tagged acylated CyaA (A-CyaA)

To express and purify recombinant acylated CyaA (A-CyaA). from *E coli,* plasmid pJR2 (Fig 3.1) was constructed that encodes the *cyaA* and *cyaC* genes. The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech, Germany); the sequence is listed in appendix I.1. *E. coli* strain XL-1 blue, were transformed with this plasmid and bacteria harbouring pJR2 were induced to express a six His-tagged acylated CyaA

3.2.1.2 His-tagged non-acylated (NA-) CyaA

To express and purify non-acylated CyaA (NA-CyaA). from *E. coli*, plasmid pJR1 (Fig 3.2) was constructed that encodes the *cyaA* gene in the absence of the *cyaC* gene. The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech); the sequence is listed in appendix I.2. *E. coli* strain XL-1 blue, were transformed with this plasmid and bacteria harbouring pJR1 can be induced to express a six His-tagged non-acylated CyaA

3.2.1.3 His-tagged enzymatically inactive (iAC-) CyaA

To express and purify CyaA with an inactive adenylate cyclase domain (iAC-CyaA) the plasmid pNM2 (Fig 3.3) was constructed that encodes the *cyaA* gene with three amino acid substitutions in the enzymatic domain that ablates enzymatic function together with the *cyaC gene.* The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech); the sequence is listed in appendix I.3. *E. coli* strain XL-1 blue, were transformed with this plasmid and bacteria harbouring the pNM2 plasmid were induced to express an acylated His^{63}→Arg / Lys^{65}→Ala / Ser^{66}→Gly (H63A/K65A/S66G) mutant of His-CyaA.

3.2.2 Expression and purification of A-CyaA.

To examine the modulation of innate and adaptive immunity by CyaA a recombinant preparation was purified from *E. coli* with particular attention paid to reducing the levels of contaminating LPS in the preparations. The plasmid pJR2, expressing 6xHis-tagged CyaA and CyaC under the control of the IPTG-inducible promoter p_{tac}, was introduced into electrocompetent *E. coli* XL1-blue cells. Bacteria harbouring the recombinant plasmid were recovered and were induced to express active CyaA that was solubilised from bacterial inclusion bodies and purified on DEAE and Ni^{++} columns. The purity of the 200 kDa CyaA protein was greater than 95% as estimated by Coomassie staining following gel electrophoresis (Fig. 3.4A) and this band was recognised by both anti-His and anti-CyaA antibodies (Fig. 3.5). The LPS content of the CyaA protein was monitored throughout the purification process. At this stage the protein preparation contained >2 ng LPS/μg protein. A number of procedures were used to attempt to remove the LPS. Passage of the protein preparation through polymyxin B conjugated columns reduced, but did not eliminate, the LPS and also resulted in significantly less protein being recovered. This suggested that CyaA was closely complexed with LPS, as has been previously reported for other RTX toxins (Czuprynski and Welch, 1995, Li and Clinkenbeard, 1999). LPS can be dissociated from proteins by the addition of EDTA to chelate calcium ions and by lowering the pH to below the pI of the protein (Petsch and Anspach, 2000). After dialysis of the protein in these conditions, the preparation of CyaA contained 220 pg LPS /μg protein. From a starting culture of 500 ml of *E. coli*, 251 μg of CyaA was recovered. This preparation was used in the adjuvant and immunomodulatory studies described in chapter 4.

3.2.3 Expression and Purification of CyaA derivatives

Recombinant CyaA and CyaA derivatives lacking acylation or deficient in adenylate cyclase activity were expressed and purified from *E. coli* to examine the role of acylation and adenylate cyclase activity in the adjuvant and immunomodulatory effects of CyaA. Acylated CyaA (A-CyaA) was purified from *E. coli* XL-1 Blue (pJR2) expressing His-CyaA and CyaC together under the control of the IPTG-inducible promoter p_{tac}. Non-acylated CyaA (NA-CyaA) was purified from *E. coli* carrying pJR1,

a similar plasmid that lacks *cyaC* (Fig. 3.4B). A CyaA protein with an inactive adenylate cyclase domain (iAC-CyaA) was generated by site directed mutagenesis of the *cyaA* gene so that it would encode a mutated protein with His^{63}→Arg, Lys^{65}→Ala and Ser^{66}→Gly (H63A, K65A and S66G) substitutions (Fig. 3.4C); Lys^{65} and His^{63} have previously been shown to be essential for the catalytic activity of CyaA (Au *et al.*, 1989, Munier *et al.*, 1992). Like the wildtype A-CyaA, NA-CyaA and iAC-CyaA were detected by Western blotting using anti-His and anti-CyaA antibodies (Fig. 3.5). Simular to wildtype CyaA, LPS co-purifies with NA-CyaA and iAC-CyaA, as they appear to form a complex, the LPS content of the two A-CyaA, NA-CyaA and iAC-CyaA preparations used was 191 and 217, 184 and 124 and 191 and 200 pg LPS/μg protein respectively. Although the LPS content has not been addressed in many studies, these concentrations are considerably lower, than those that have been reported by others (Macdonald-Fyall *et al.*, 2004, Prior *et al.*, 2002).

3.2.4 Biochemical properties of recombinant CyaA proteins.

3.2.4.1 Acylation of CyaA

Post-translational acylation of CyaA by the specific lysine acyl-transferase CyaC adds a palmitoyl group to the Lys^{983} residue of CyaA by amide linkage. In recombinant preparations, Lys^{860} can also become palmitoylated by CyaC. This modification has been reported to be important for the interaction of CyaA with target cells. The presence of palmitic fatty acid acylation on A-CyaA and iAC-CyaA and the absence of palmitic fatty acid acylation NA-CyaA, was confirmed by inducing protein expression in *E. coli* in the presence of [^{14}C(U)] palmitic acid. Fluorography was employed to visualise the radio-labelled proteins and the results demonstrated that the A-CyaA and iAC-CyaA preparations were modified by post-translational acylation by the co-expressed accessory protein CyaC, whereas CyaA expressed in the absence of CyaC (NA-CyaA) did not become palmitoylated (Fig. 3.6).

3.2.4.2 *In vitro* CyaA enzymatic activity

The N-terminal of CyaA contains an enzymatic domain that converts ATP into cAMP and required eukaryotic calmodulin to be fully active (Wolff *et al.*, 1980). To confirm that the recombinant proteins exhibited the expected enzymatic activity the enzymatic activity of CyaA and CyaA derivatives were measured using a commercial competitive cAMP ELISA kit (Amersham Biosciences, UK). *In vitro* cell-free assays of the adenylate cyclase enzyme function of the preparation of A-CyaA described in section 3.2.2 and used in the experiment in chapter 4 demonstrated that the protein was enzymatically active with a specific activity of 26.6 ± 0.8 μmol cAMP/min/mg.

Examination of the specific activity of the preparations described in section 3.2.3 demonstrated that both A-CyaA and NA-CyaA were enzymatically active, exhibiting specific activities of 126.9 ± 29.9 and 113.0 ± 11.3 μmol cAMP/min/mg respectively (Fig. 5.1), these are within the spectrum of those reported from other laboratories (Gray *et al.*, 1998, Khelef *et al.*, 2001, Macdonald-Fyall *et al.*, 2004, Masin *et al.*, 2004), while the iAC-CyaA was devoid of enzyme activity confirming that the site directed mutagenesis ablated the enzymatic function. The disparity between the two A-CyaA preparations was attributed to the use of polymyxin B conjugated columns to remove LPS during the purification of the first preparation, which appeared to reduce the enzymatic activity of the preparation.

3.2.4.3 Intracellular cAMP accumulation.

The enzymatic activity of CyaA is important for the function of CyaA during *B. pertussis* infection (Gross *et al.*, 1992). In addition, it has been reported that blocking the action of CyaA with small molecule inhibitors of cAMP blocks its ability to induce maturation of human DC (Bagley *et al.*, 2002b). To confirm that the recombinant protein preparations possessed the expected enzymatic activity and to assess their activity inside the cell each preparation was tested for its ability to upregulate intracellular cAMP in J774 macrophages. The preparation of A-CyaA (used in the experiments in chapter 4) was tested at a concentration of 1 μg/ml and was able to increase the intracellular concentration of cAMP 80-fold in J774 macrophages (72.8 ± 2.9 vs 0.9 ± 0.1 pmol cAMP/10^6 cells), showing that the protein has the ability to target

and enter eukaryotic cells. The maximal cAMP concentration was reached within 30 min and was maintained for at least 24 h.

The preparations of A-CyaA, NA-CyaA and iAC-CyaA (used in the experiments in chapters 5, 6 and 7) increased intracellular cAMP concentrations in macrophages to 1457.0 ± 128.6, 708.5 ± 17.7 and 0 pmol cAMP/10^6 cells respectively while untreated cells remained at background levels. A commercial preparation of CyaA purchased from List Biological Laboratories, Inc. (CA, USA) was tested in parallel with the recombinant CyaA and CyaA derivatives. The specific activity of this commercial preparation was found to 150 μmol cAMP / mg / min, while the documentation from the supplier listed the activity as 600 μmol cAMP / mg / min using a different assay. This suggests that the assay used in the present studies may under-estimate the enzymatic activity of the CyaA and CyaA derivative preparations.

3.2.4.4 Cell lysis

In addition to specifically binding innate immune cells that express the CD11b/CD18 integrin (Guermonprez *et al.*, 2001), CyaA can interact with the membranes of a variety of eukaryotic cells through the hydrophobic domain located in the C-terminal of the protein (Khelef *et al.*, 2001, Lee *et al.*, 1999, Otero *et al.*, 1995, Westrop *et al.*, 1997). This region was also shown to form pores in the cell membrane, resulting in potassium efflux, altered cell metabolism and ultimately cell lysis (Gray *et al.*, 1998). I tested the ability of the recombinant preparations of CyaA and CyaA derivatives to induce the lysis of J774 macrophages. A-CyaA (used in chapter 4) induced lysis of up to 10% of macrophages at concentrations of 10 μg/ml, measured by LDH release. However, a concentration of 1 μg/ml induced less than 1% cell lysis even after 24 h. Therefore, an concentration of 1 μg/ml was chosen for future experiments designed to investigate the immunomodulatory function of CyaA. This is a concentration that induces a large increase in intracellular cAMP, without affecting cell viability.

When tested at 10 μg/ml, the preparations of A-CyaA, NA-CyaA and iAC-CyaA (used in chapters 5,6 and 7) induced 27.0% ± 5.8, 4.6% ± 4.1 and 13.1% ± 5.2 lysis of macrophages respectively; the back-ground lysis of untreated cells was 0.3% ± 0.3.

Lysis was not observed with any of the preparations when cells were incubated with 1 μg/ml of each of the CyaA preparations and this was the concentration that was used in future immunomodulatory experiments.

3.3 Discussion

To investigate the immunomodulatory properties of CyaA, a preparation free of contaminating immunomodulatory molecules was required. A recombinant N-terminal 6xHis-tagged CyaA was produced in *E. coli* from plasmid pJR2, constructed to encode the *cyaA* gene and the *cyaC* gene cloned from the genomic DNA of *B. pertussis*. CyaC is the specific accessory protein required for post-translational modification of CyaA. The sequence of the cloned genes and their position within the expression plasmid were confirmed by sequencing. CyaC is a specific lysine acyl-transferase that post-translationally modifies CyaA by the addition of a palmitoyl fatty acid by amide linkage (Hackett *et al.*, 1994, Havlicek *et al.*, 2001). In both *B. pertussis* (Basar *et al.*, 2001, Hackett *et al.*, 1994l) and *E. coli* recombinant systems (Hackett *et al.*, 1995, Havlicek *et al.*, 2001) CyaA is acylated at Lys^{983}. While CyaA expressed in *E. coli* expression systems is also acylated at Lys^{860} (Hackett *et al.*, 1995, Havlicek *et al.*, 2001). The acylation of CyaA expressed from plasmid pJR2 was confirmed by inducing the expression of the proteins from *E. coli* in the presence of ^{14}C-lableled palmitoylic acid followed by fluorography to visualise the radiolabelled CyaA. This post-translational acylation is important for the interaction of CyaA with target cells. It has been published that acylation of CyaA is essential for the interaction of CyaA with the membranes of target cells and to form haemolytic pores (Heveker *et al.*, 1994). However, it has been reported that non-acylated CyaA does interact with cells expressing CD11b although this does not result an increase in cAMP levels in the target cells (El-Azami-El-Idrissi *et al.*, 2003).

To investigate if acylation plays a role in the modulation of immunity by CyaA, a recombinant N-terminal 6xHis-tagged non-acylated form of CyaA (NA-CyaA) was generated. The plasmid pJR1 was constructed to encode the *cyaA* gene in the absence of the *cyaC* gene and the position and sequence of the cloned gene was confirmed by sequencing. *E. coli* harbouring the pJR1 plasmid were induced to express CyaA in the absence of CyaC and NA-CyaA was purified. The lack of acylation of NA-CyaA was confirmed by fluorography after induction of NA-CyaA expression in the presence of ^{14}C-labeled palmitic acid.

CyaA encodes an enzymatic domain in the N-terminal of the protein that converts ATP to cAMP in a calmodulin dependant manner. The Lys^{65} and His^{63} residues have previously been shown to be essential for this catalytic activity of CyaA (Au *et al.*, 1989, Munier *et al.*, 1992). To investigate the role of the enzymatic activity in the immunomodulatory effects of CyaA, a mutant with His^{63}→Arg, Lys^{65}→Ala, Ser^{66}→Gly (H63A/K65A/S66G) amino acid substitutions was constructed by site-directed mutagenesis of the *cyaA* gene. The plasmid pNM2 was constructed to express a 6xHis-tagged H63A/K65A/S66G mutant of CyaA together with CyaC to produce an acylated derivative of CyaA with an inactive adenylate cyclase domain (iAC-CyaA). The position and sequence of the cloned genes, together with the mutated bases were confirmed by sequencing. iAC-CyaA was purified from *E. coli* harbouring the pNM2 plasmid and acylation of iAC-CyaA was demonstrated with radiolabelled palmitoylic acid by fluorography. Protein preparations of CyaA and CyaA derivatives used in all immunomodulatory experiments were greater than 95% pure as determined by staining on SDS-PAGE gels and their identity confirmed by Western blot analysis using antibodies specific for CyaA and the 6xHis-tag at the N-terminal of each protein.

The purification regime employed to purify the recombinant CyaA and CyaA derivatives went to great lengths to remove LPS from each preparation. LPS is a major structural component of the cell wall of Gram-negative bacteria and is a powerful immunomodulatory molecule. LPS, through TLR4, promotes inflammatory immune responses including the production inflammatory cytokines such as IL-12p70 and TNF-α from innate immune cells and promotes the differentiation of adaptive Th1 cells and IFN-γ production. The role of LPS co-purified with immunomodulatory molecules has not been addressed in most studies with CyaA. The conclusion that LPS contaminating a protein sample does not play a role in the immune response induced by that preparation because denaturing that protein sample by boiling abrogates the observed response does not address the possibility of co-operative effects between the protein and LPS. Even low to almost undetectable levels of LPS co-purified with proteins can alter the induced immune response through synergy or suppression. Most work that has been published on the biological activity of native or recombinant CyaA has failed to address the issue of LPS contamination. Rarely has the level of LPS in the protein

preparations even been reported. One study, by Prior *et al.* (Prior *et al.*, 2002), which directly addressed the toxicity of recombinant CyaA preparations, reported that 8 different CyaA preparations from two different laboratories had high levels of LPS contamination (3000-1,800,000 EU/mg CyaA). It is exceedingly likely that much of the previously published data on the immunological activity of adenylate cyclase toxin is complicated by the activity of contaminating LPS. CyaA is a member of the RTX family of toxins and is a hydrophobic molecule, which facilitates LPS binding to the toxin. Furthermore, it has been shown that RTX toxins in particular bind LPS tightly (Czuprynski and Welch, 1995, Li and Clinkenbeard, 1999). Therefore every preparation of CyaA either purified from *B. pertussis* or as a recombinant protein in *E. coli* contains co-purified LPS unless specific steps to remove or reduce this LPS content were performed and verified by highly sensitive LPS detection assays. LPS has potent biological activities in many systems, including but not confined to immunomodulation and lytic activity. To address this, the purification protocols used here are designed to reduce the LPS content. In fact, all of the protein preparation purified and used in the experiments in the following chapters contained less than 2.2 EU of LPS/ μg CyaA. Although these are exceptionally low amount of LPS, it is enough to activate DC in synergy with other bacterial derived molecules, such as CT and LT (Lavelle *et al.*, 2003, Lavelle *et al.*, 2004). For this reason, the CyaA preparations were tested, where possible, in assay systems that negate the effects of very low levels of LPS remaining in the protein preparations.

The catalytic domain of CyaA converts ATP to cAMP, this enzymatic activity plays an important role in the activity of CyaA during *B. pertussis* infection (Gross *et al.*, 1992). Interaction of CyaA with eukaryotic cells results in the production of supraphysiological levels of intracellular cAMP, cAMP accumulation has also been shown to activate human DC (Bagley *et al.*, 2002b). Therefore, the upregulation of cAMP may be important for the immunomodulatory activity of CyaA. To measure the intracellular cAMP activity of the recombinant A-CyaA, J774 macrophages were treated with A-CyaA. As expected, this resulted in an upregulation of intracellular cAMP, with 1457.0 ± 128.6 pmol cAMP/10^6 cells (72.8 pmol cAMP/10^6 cells for an earlier batch) measured, while untreated cells had less than 1 pmol cAMP/10^6 cells.

Surprisingly, NA-CyaA also enhanced intracellular cAMP concentrations in macrophages, with 708.5±17.7 pmol cAMP/10^6 cells detected after treatment with NA-CyaA for 60 minutes. This finding conflicts with previous reports, which indicated that NA-CyaA does not interact with cells and that post translational modification by CyaC is required for the translocation of the catalytic AC-domain through the target cell membranes, as well as the formation of haemolytic cation-selective CyaA channels (Barry *et al.*, 1991, Basar *et al.*, 2001, El-Azami-El-Idrissi *et al.*, 2003, Heveker *et al.*, 1994, Hewlett *et al.*, 1993, Rogel *et al.*, 1989). As expected, treatment of J774 macrophage cells with iAC-CyaA, which is devoid of enzymatic activity, did not increase intracellular cAMP levels (Ehrmann *et al.*, 1992).

The specific activities of the purified recombinant A-CyaA and the NA-CyaA derivative preparations were measured in an *in vitro* cell free assay, and specific activities of 126.9 ± 29.9 and 113.0 ± 11.3 μmol cAMP/min/mg respectively were recorded. The iAC-CyaA derivative, which has the His^{63}→Arg / Lys^{65}→Ala / Ser^{66}→Gly (H63A/K65A/S66G) substitutions in the enzymatic domain, has no adenylate cyclase activity. The adenylate cyclase activities of A-CyaA and NA-CyaA detected are at the lower end but still well within the spectrum of activities that are reported by other laboratories for CyaA.

However, it is possible that the stringent methods used here to purify the CyaA preparations free of LPS may have been quite harsh and resulted in some changes to protein structure that may have resulted in a reduction in enzymatic activity. Nevertheless, certain of the conditions used during the purification of CyaA, specifically the concentrations of Urea and EDTA within the buffers, are similar to those used by other laboratories (Gao *et al.*, 2002, Hewlett *et al.*, 1991, Sebo *et al.*, 1991). Reducing the pH is a standard procedure for eluting protein from Nickel or other affinity columns and was used in the purification of the His-tagged CyaA proteins. Indeed, changes in buffer urea concentrations during the dialysis procedure to remove LPS contamination of the protein were done gradually in order to avoid dramatic changes to conditions and to prevent sudden changes to protein conformation.

It is more likely that the differences in adenylate cyclase activity reported by other laboratories is more likely to reflect laboratory to laboratory variation in the

assays. Comparisons of intracellular cAMP with those of other laboratories are not straightforward and are complicated by the assays systems, different methods of reporting the data and cell types used for intracellular cAMP induction. Comparing the specific activities reported by different laboratories reveal that they vary greatly. The highest activity that was reported in a paper is from the Hewlett laboratory (1770 μmol cAMP / mg CyaA / min) (Gray *et al.*, 1998) and this was significantly higher than that reported in other papers, including 589 μmol cAMP /mg CyaA / min from the same lab (Hewlett *et al.*, 1989) and 330 μmol cAMP / mg CyaA / min from the Guiso laboratory (Khelef *et al.*, 2001) and 194 μmol cAMP / mg CyaA / min from the Coote laboratory (Macdonald-Fyall *et al.*, 2004). However, the methods used to determine enzyme activity in the different laboratories are not identical and a variety of factors, including the temperature of the reaction, can affect the activity recorded. In the paper by Grey *et al.* (Gray *et al.*, 1998) activities of 1770 and 117 μmol cAMP /mg CyaA / min are reported for assays performed at 37^{0}C and 0-2^{0}C respectively. The activity of 589 μmol cAMP / mg CyaA / min in the paper by Hewlett *et al.* (Hewlett *et al.*, 1989) was based on a reaction temperature of 30°C. Macdonald-Fyall *et al.* (Macdonald-Fyall *et al.*, 2004) reported activities of 194 or 453 μmol cAMP / mg CyaA / min for the same preparation of CyaA using two different assays. When a commercially available preparation of CyaA was tested in parallel with the recombinant protein purified here, using the same assay conditions, it was found to have a specific activity of 150 μmol cAMP / mg / min, which is similar to the activities of the recombinant A-CyaA and NA-CyaA generated in these studies, but substantially less than the activity recorded by the suppliers of 600 μmol cAMP / mg / min for this batch using a different assay. The cAMP experiments used here were performed at 30^{0}C while others have not reported the temperatures or in some cases given any details of the assay employed. Therefore it is very difficult to make direct comparisons of activity based on data reported in different laboratories

Interestingly, while other laboratories have reported high levels of lysis in cells treated with CyaA. The recombinant A-CyaA purified here and used in the subsequent studies demonstrated maximum lysis of between 30-40% at the highest concentration used to treat cells (10 μg/ml). In studies published by other groups, high levels of lysis

were recorded for treatments with lower concentrations of CyaA. This may reflect the lower levels of contaminating LPS in the recombinant protein preparations used in the present studies. Evidence in support of this conclusion is shown later in chapter 5 (in Fig 5.2), where chelating LPS with polymyxin B significantly reduced the lytic activity of CyaA. While 4.6 ± 4.1% lysis of macrophages induced by NA-CyaA is not significantly different from the 0.3 ± 0.3% lysis measured for untreated cells is not significant, data presented later in chapter 5 shows that the NA-CyaA preparations induces cell lysis at high concentrations of 10 μg/ml only. This is not in agreement with other published work, which has reported that post-translational acylation of CyaA is required for it to bind cells and to form haemolytic channels in the membranes of target cells (Barry *et al.*, 1991, Basar *et al.*, 2001, Hackett *et al.*, 1994). However when LPS is chelated with polymyxin B this lysis by NA-CyaA was abrogated. Indicating that the lysis by NA-CyaA may at least in part be due to residual LPS present in the purified preparation. This, together with the reduction in lysis induced by A-CyaA in the presence of polymyxin B, suggests that LPS, especially at relatively high concentrations can synergise with CyaA to cause cell lysis. In fact, LPS itself can cause cell death (Hull *et al.*, 2002). These results may help explain the lower levels of cell lysis and cytotoxicity observed with the A-CyaA preparation compared to that reported from other laboratories, who did not report the levels of contaminating LPS in the CyaA preparations. Indeed the one study that compared CyaA preparations from different laboratories reported up to $1.8x10^3$ times the amount of LPS present in the CyaA preparations used in these studies (Prior *et al.*, 2002).

It is important to have a pure preparation of a molecule to investigate how it modulates the immune response. The purification regime used to purify CyaA and the CyaA derivatives yields a preparation that contains the lowest amount of LPS reported for recombinant CyaA from any laboratory to date. While the recombinant preparations of A-CyaA retain the enzymatic activity it was used at a concentration that induces maximal cAMP production in target cells but not lysis. In addition, to fully recognise the low level of LPS co-purified with each protein preparation the experiments investigating the immunomodulatory effects of CyaA in the following chapters were carried out in systems that negate the effects of low amounts of LPS.

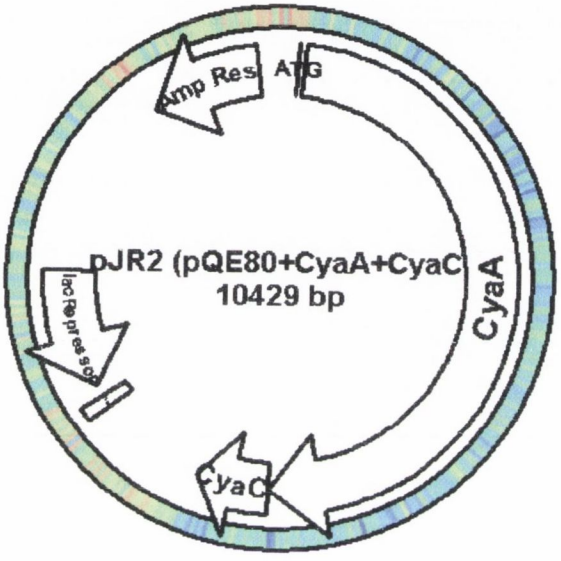


Figure 3.1: pJR2.
Plasmid pJR2 which encodes CyaA and CyaC was constructed to express His-tagged acylated CyaA in *E. coli*,

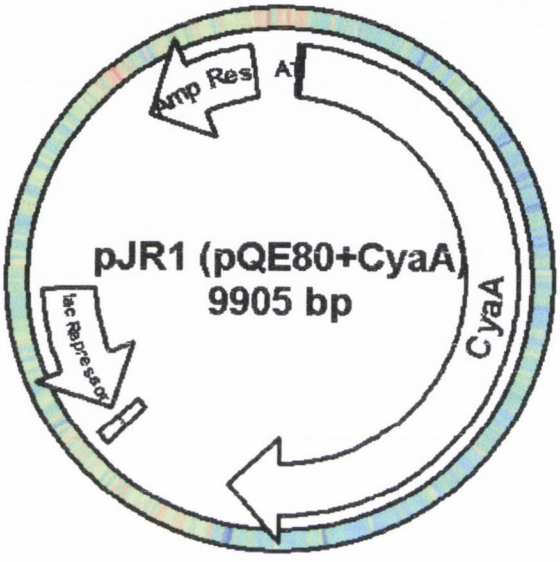


Figure 3.2: pJR1.
Plasmid pJR1 which encodes CyaA was constructed to express His-tagged non-acylated CyaA in *E. coli*

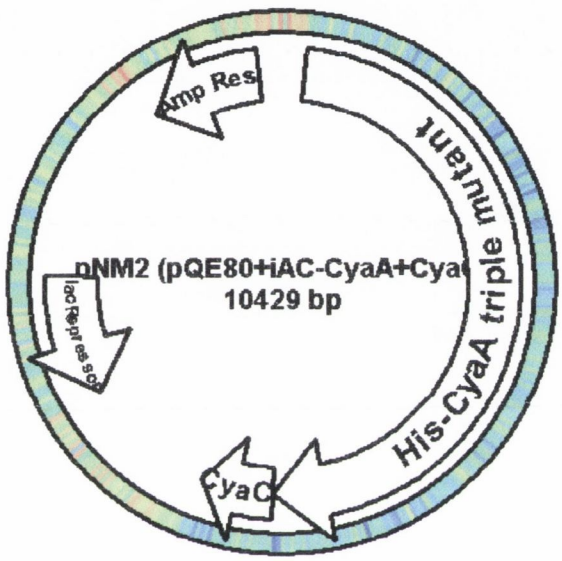


Figure 3.3: pNM2
Plasmid pNM2 which encodes a H63A/K65A/S66G CyaA mutant was constructed to express His-tagged acylated CyaA with an inactive adenylate cyclase domain in *E. coli*.

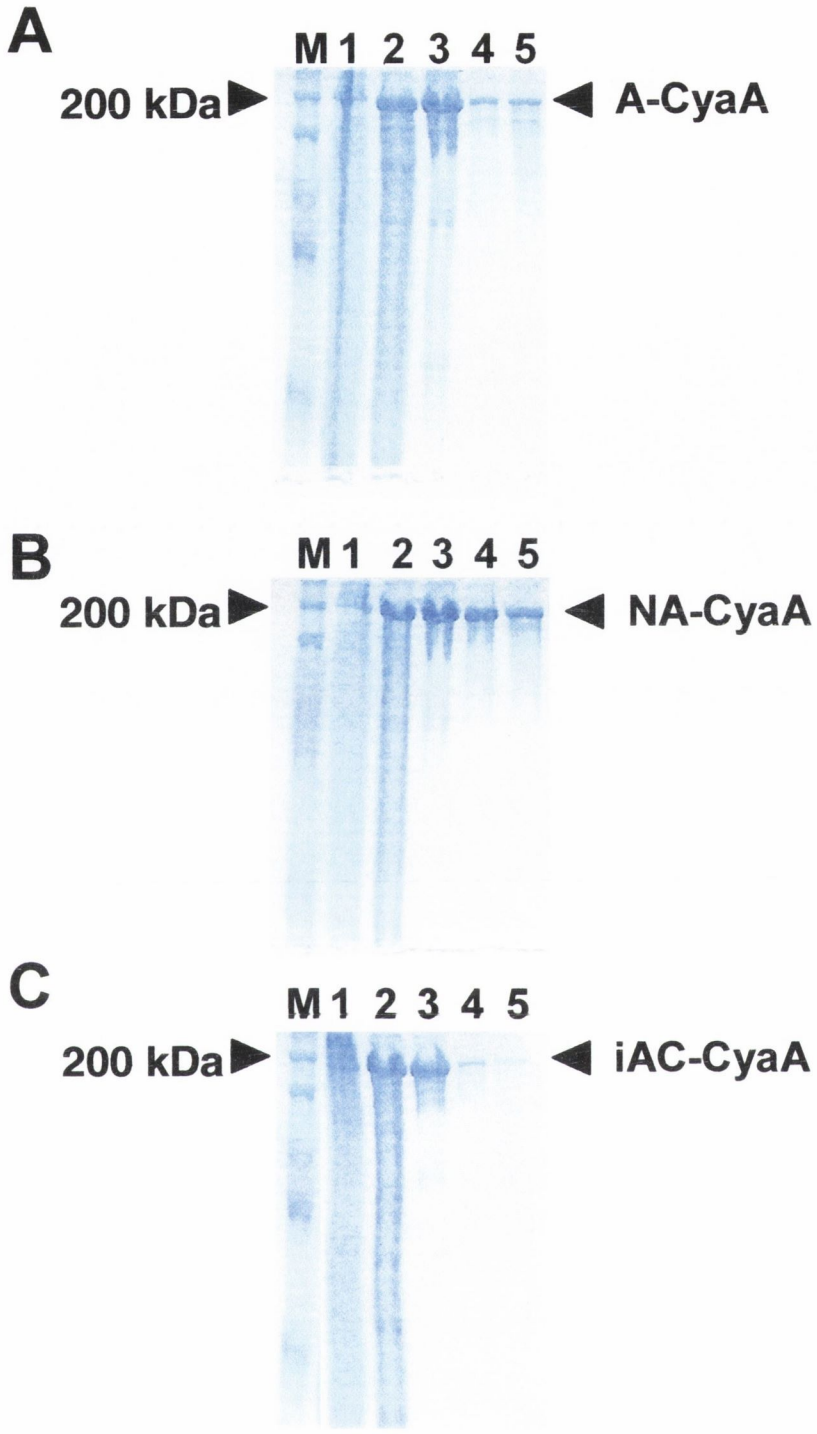


Figure 3.4: Protein purification of His-tagged CyaA and CyaA derivatives. *E. coli* harbouring the plasmids pJR2, pJR1 and pNM2 were induced to express A-CyaA (A), NA-CyaA (B) and iAC-CyaA (C) respectively. During the purification protocol, 10 µl of the recovered protein was retained after each stage and visualised by SDS-PAGE. M; Molecular weight marker, 1; Whole bacterial cell lysate, 2; Insoluble protein, 3; DEAE column elute, D4 His-column elute, 5; Dialysate.

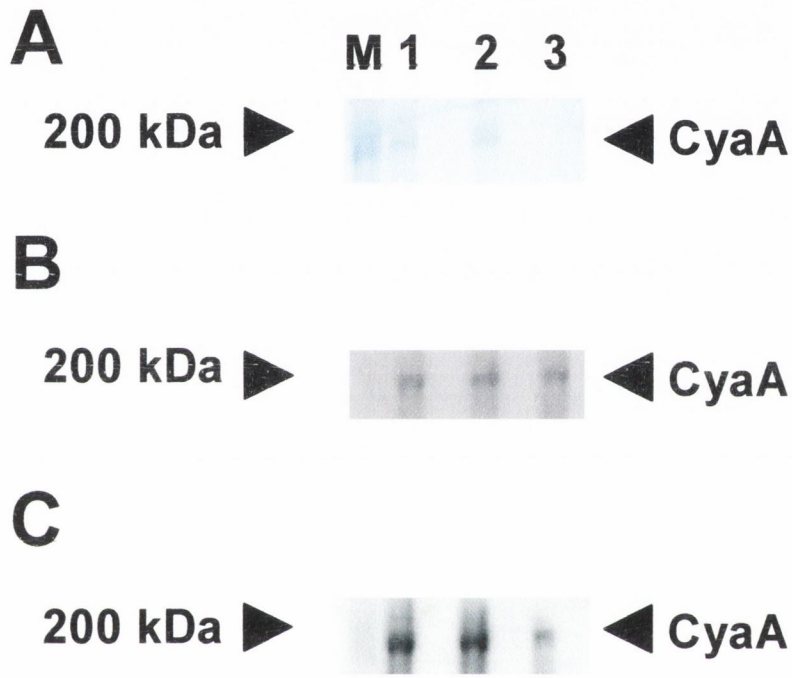


Figure 3.5: Western blot of CyaA and CyaA derivatives
Highly purified A-CyaA (1), NA-CyaA (2) and iAC-CyaA (3) were separated by SDS-PAGE, stained with Gel-code Blue reagent (A) or transferred to nitrocelulose membrane and probed with anti-CyaA (A) and anti-His (B) antibodies. M; Molecular weight marker.

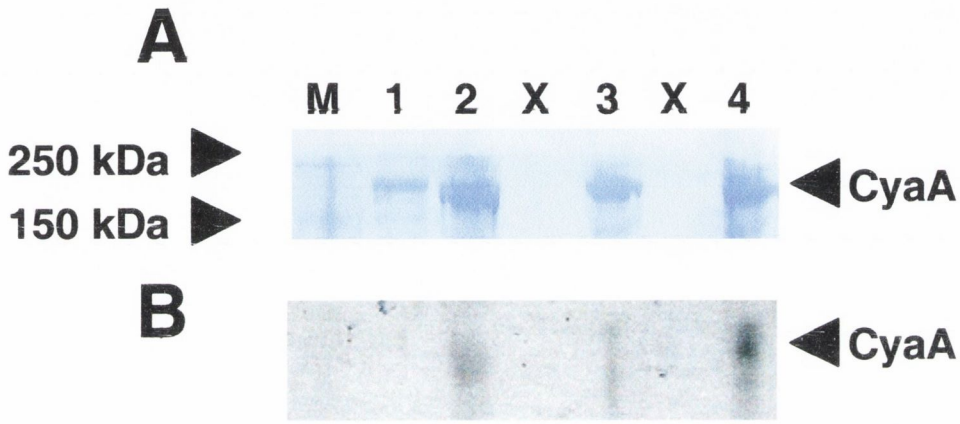


Figure 3.6 Acylation of CyaA and CyaA derivatives
E. coli harbouring the plasmids pJR2, pJR1 and pNM2 were induced to express A-CyaA, NA-CyaA and iAC-CyaA respectively, by the addition of 1 mM IPTG for 15 minutes in medium supplemented with [^{14}C(U)] palmitic acid. Bacteria were pelleted and washed in fresh medium before sepertion on 7% SDS-PAGE gel (A) and visualised with Gel-code Blue reagent before flourographic analysis to visualise radiolabelled proteins (B). Lane 1, highly purified CyaA (not radioactively labeled); lane 2, A-CyaA; lane 3, NA-CyaA; lane 4, iAC-CyaA; M, molecular weight marker; X, empty lane.

CHAPTER 4

CyaA SYNERGISES WITH LPS TO PROMOTE INNATE IL-10 PRODUCTION AND ENHANCES THE INDUCTION OF Th2 AND REGULATORY T CELLS

4.1 Introduction

The identification of invading micro-organisms by the immune system is crucial for the prevention of disease and clearance of the pathogen. Cells of the innate immune system, including macrophages and especially dendritic cells (DC), are important sentinel cells that direct the differentiation of naïve $CD4^+$ T cells into functionally distinct Th1, Th2 or regulatory T (Tr) cell subtypes (McGuirk and Mills, 2002).The products and components of micro-organisms and other parasites have conserved molecular features that are recognised by receptors on these antigen presenting cells (APC) of the innate immune system. Binding of these conserved pathogen associated molecular patterns (PAMPs) to pathogen recognition receptors (PRRs), such as Toll-like receptors (TLR), complement receptors and integrins, results in the activation of the immature DC and is accompanied by its maturation and homing to the lymph nodes, where the mature DC presents antigen to naïve T cells (Akira, 2003, McGuirk *et al.*, 2002). This crucial step in turn activates the antigen specific adaptive immune response needed to eradicate the pathogen and generate immunological memory against further infections by the micro-organism. This adaptive immunity hinges on the activation of naïve antigen-specific T cells by DC. In their immature form DC reside in sites of the body prone to infection, constantly sampling their environment by phagocytosis, endocytosis and micropinocytosis. Exogenous antigen is phagocytosed and processed by DC and presented to the naïve T cell as MHC class-II –peptide complexes on the surface of the DC. Ligation of the T cell receptor to the MHC class-II antigen complex is accompanied by binding of co-stimulatory molecules upregulated on the surface of the activated DC to their respective ligands on the surface of the T cell. These two signals in concert with cytokines produced by the DC drive the proliferation of the T cell and the polarisation into a Th1, Th2 or Tr1 phenotype (Kapsenberg, 2003, McGuirk and Mills, 2002, Mills, 2004, Moser and Murphy, 2000, O'Garra and Arai, 2000).

DC differentiate between pathogens, inducing the appropriate type of T cell response necessary to combat the infection. Certain pathogen derived molecules, including lipopolysaccharide (LPS), unmethylated CpG motifs in DNA, dsRNA and pertussis toxin (PT), activate DC that drive the differentiation of Th1 cells (de Jong *et*

al., 2002, Hemmi *et al.*, 2000). In contrast, products of helminth parasites (Whelan *et al.*, 2000), cholera toxin (CT) (Gagliardi *et al.*, 2000), and yeast (d'Ostiani *et al.*, 2000, Edwards *et al.*, 2002) activate DC that drive the differentiation of Th2 cells. Finally, filamentous haemagglutinin (FHA) from *Bordetella pertussis* (McGuirk *et al.*, 2002) and CT (Lavelle *et al.*, 2003) activate DC that promote the differentiation of IL-10-secreting type 1 Tr (Tr1) cells. Cytokines, including IL-4, IL-6, IL-10 and IL-12, secreted by innate immune cells in response to pathogen-derived molecules, play a critical role in regulating the differentiation of naïve $CD4^+$ T cells into distinct T cell subtypes (McGuirk *et al.*, 2002, Mendel and Shevach, 2002, Moser and Murphy, 2000). Th1 cells produce large amounts of IFN-γ and are essential to combat infections by bacteria. Th2 cells, which secrete IL-4, IL-5 and IL-10, are important in immunity against helminth parasites, fungi, and intracellular bacteria. IL-10 secreting T regulatory type 1 (Tr1) cells play a role in preventing autoimmune diseases and allergy as well as preventing excessive damage by the host inflammatory response to a pathogen. In turn, the cytokines secreted by these $CD4^+$ T cells are important in activating and recruiting phagocytic cells to the site of infection. In addition, $CD4^+$ T cells help mature B cells into plasma cells through the secreted cytokines, which also promote the isotype switching of the secreted antibodies. These antibodies play an important role in opsonising the pathogen, enabling the phagocytic cell to eliminate the micro-organisms.

The Gram-negative bacterium, *B. pertussis*, causes whooping cough, a severe and prolonged infection in young children. A number of virulence factors of *B. pertussis*, including PT, LPS, FHA and adenylate cyclase toxin (CyaA), that are essential for bacterial colonisation, can modulate host immune responses (Mills, 2001). Bacteria deficient in CyaA are less pathogenic in mice and fail to stimulate inflammatory cell recruitment into murine lungs (Goodwin and Weiss, 1990, Gross *et al.*, 1992, Harvill *et al.*, 1999, Khelef *et al.*, 1994, Weiss *et al.*, 1984). CyaA has been shown to subvert immune responses to *B. pertussis* by interfering with chemotaxis, phagocytosis and superoxide production in host cells through the generation of supraphysiological levels of cAMP (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987). Furthermore, CyaA causes lysis and cytotoxicity in a variety of

cells, albeit with various degrees of efficiency (Hewlett *et al.*, 1993, Khelef *et al.*, 2001, Westrop *et al.*, 1997) and induces apoptosis in macrophages (Gueirard *et al.*, 1998, Khelef *et al.*, 1993). CyaA is encoded by the *cyaA* gene and is post-translationally modified through palmitoylation of K_{983} (Hackett *et al.*, 1994) by the product of the *cyaC* gene (Barry *et al.*, 1991). Although *E. coli* expressed CyaA can also be palmitoylated at K_{860} (Hackett *et al.*, 1995), the palmytiolation of K_{983} seems crucial to the activity of CyaA (Basar *et al.*, 2001). The C-terminal 1306 amino acids contain a series of nonapeptide repeats involved in calcium binding (Rose *et al.*, 1995), similar to the repeat in toxin (RTX) family of exotoxins which have haemolytic and immune stimulatory properties (Coote, 1992). The N-terminal 400 amino acids contain the catalytic domain (Sakamoto *et al.*, 1992) that converts ATP to cAMP (Confer and Eaton, 1982). Upon cell binding, the enzymatic domain is delivered into the cytosol where it must bind eukaryotic calmodulin to become enzymatically active (Wolff *et al.*, 1980). This translocation is calcium and temperature dependant and also depends on the plasma membrane potential (Otero *et al.*, 1995).

The invasive nature of CyaA has been employed to deliver antigenic peptides into the endogenous route of antigen processing for presentation to MHC class I-restricted $CD8^+$ T cells (Fayolle *et al.*, 1996). In addition, strong $CD8^+$ CTL responses were detected in mice immunised with recombinant CyaA carrying CTL epitopes from LCMV and HIV-1 gp120 (Fayolle *et al.*, 1996). Mice immunised with a toxoid carrying the LCMV $CD8^+$ T cell epitope were efficiently protected from subsequent lethal challenge with a virulent strain of lymphocytic choriomeningitis virus (LCMV) (Fayolle *et al.*, 2001, Saron *et al.*, 1997). A genetically detoxified version carrying the same epitope yet unable to convert ATP into cAMP, stimulated equally efficient $CD8^+$ T cell responses and protective immunity, demonstrating the usefulness of CyaA as a delivery vehicle for CTL epitopes (Fayolle *et al.*, 2001). Indeed, therapeutic immunity against melanoma tumors expressing ovalbumin was induced in mice using CyaA carrying the $CD8^+$ epitope from ovalbumin (Fayolle *et al.*, 1999). Recently it has been shown that an enzymatically inactive CyaA could also deliver an epitope into the MHC class II processing pathway for activation of $CD4^+$ cells (Loucka *et al.*, 2002). In addition, CyaA has been shown to enhance antibody levels to co-administered

ovalbumin (Hormozi *et al.*, 1999) and to promote Th1 responses to an expressed viral epitope (Dadaglio *et al.*, 2000). The adjuvant activity of CyaA may reside in its ability to activate cells of the innate immune system through the upregulation of cAMP (Gentile *et al.*, 1990) and/or the binding to the CD11b/CD18 $\alpha_M\beta_2$ integrin (Guermonprez *et al.*, 2001), expressed on innate immune cells, including macrophages and DC. Interestingly, it has been published that acylation of CyaA is required for tight interaction of the toxin with cells expressing CD11b, while the catalytic domain is not required for this interaction (El-Azami-El-Idrissi *et al.*, 2003). It has been reported that CyaA can promote maturation and suppress inflammatory cytokine production by human monocyte derived DC and that this is mediated by cAMP (Bagley *et al.*, 2002b).

This study examines the adjuvant and immunomodulatory activity of CyaA, paying particular attention to a possible contribution of LPS, known to be closely associated with RTX molecules (Czuprynski and Welch, 1995, Li and Clinkenbeard, 1999). The hypothesis that CyaA may act as an adjuvant to enhance specific subsets of $CD4^+$ T cells by promoting activation of DC is addressed. The findings demonstrate that CyaA has adjuvant activity, promoting Th2 and Tr1 responses, with significant enhancement of antigen-specific IL-10 producing T cells. This effect appears to be mediated in part by its ability to activate cells of the innate immune system, including DC. However, the data also reveal that CyaA can synergise with and modulate TLR4-mediated responses of DC to LPS.

4.2 Results

4.2.1 CyaA generates Th2 and Tr1 cells to a co-injected antigen.

To examine the adjuvant properties of CyaA, mice were immunized s.c. in the hind footpad with the model antigen keyhole limpet hemocyanin (KLH; 5 μg), alone or with CyaA (1 μg). Seven days after immunization, mice were sacrificed and lymph node cells were re-stimulated with antigen (KLH 2-50 μg/ml) *in vitro*. Cytokine concentrations were determined in supernatants removed after 3 days and proliferation

was assessed after 4 days. Immunization with KLH alone induced weak cellular immune response; proliferation and IL-4 production was slightly enhanced over that observed in mice immunized with PBS (Fig. 4.1). In contrast, significant antigen-specific proliferation was observed in re-stimulated lymph node cells from mice immunized with KLH and CyaA. Furthermore, significantly higher concentrations of KLH-specific IL-10 and IL-5 were detected in supernatants from lymph node cells from mice immunized with CyaA and KLH compared with mice immunized with KLH alone. Production of IL-4 and IFN-γ was also enhanced, but the difference between mice that received KLH alone and KLH with CyaA was in most cases not significant. Substantially higher levels of IFN-γ were secreted by lymph nodes cells from mice immunized with KLH and CyaA when polyclonally stimulated with PMA and anti-CD3 (Fig. 4.1). In contrast and consistent with previous work, lymph node cells from mice immunized with KLH in the presence of CpG-ODN secrete 20 ng/ml of IFN-γ in response to restimulation with KLH in vitro (Fig. 4.2), which is at least 40 fold higher than that observed with KLH and CyaA (Fig 4.1). A similar pattern of cytokine secretion was observed 7 days after a booster immunization with significantly more IL-10 and IL-5 produced by cells from mice immunised with KLH and CyaA than from mice immunised with KLH alone (Fig. 4.3).

The cytokine profile of antigen-stimulated lymph node cells suggested that CyaA enhanced Th2 and/or Tr1 cells to co-administered antigens. In order to confirm this finding, KLH-specific $CD4^+$ T cell lines and clones were generated from mice immunized with KLH in the presence of CyaA. Each of the T cell lines examined secreted high levels of IL-10 and lower levels of IL-5, which is the defined cytokine profile of Tr1 cells, and a smaller number also secreted IL-4, characteristic of Th2 cells (Fig. 4.4A). IFN-γ production was detectable in 6 of 10 T cell lines examined and at high levels in only one of these T cell lines (Fig. 4.4A). In contrast, the majority of T cell lines generated from mice immunized with KLH in the presence of CpG-ODN secrete IFN-γ in the concentration range 30-50 ng/ml, with very low or undetectable IL-4 and IL-5 (Fig. 4.5). Higher levels of IFN-γ production from T cell lines from mice immunised with KLH in the presence of CpG-ODN have also been reported (Lavelle *et al.*, 2003). Four of the T cell lines generated from mice immunized with KLH and

CyaA, (2 that secrete IFN-γ and 2 that did not secrete this cytokine) were cloned and cytokine production by T cell clones from one T cell line is shown in Fig. 4.4B. These KLH-specific T cell clones secreted IL-5 and IL-10, or IL-4, IL-5 and IL-10 but undetectable IFN-γ, profiles characteristic of Tr1 and Th2 cells respectively. These findings demonstrate that CyaA promotes the induction of Th2 and Tr1-type cells specific for the co-administered antigen, and that Th1-type T cells may also be generated, but at lower frequency

4.2.2 CyaA enhances IgG1 responses to co-administered antigen.

The adjuvant effect of CyaA for antibody responses to co-injected antigen was determined by assessing KLH-specific IgG and IgG subclasses in mice immunized with KLH alone or with CyaA was examined. Significantly higher levels of KLH-specific IgG1 were found in the serum of mice immunized with KLH and CyaA compared with mice that received antigen alone. In contrast, CyaA did not enhance IgG2a levels above those observed in mice immunized with KLH alone (Fig. 4.6A). Following a second immunization the serum IgG titres were increased over those observed after a single immunization and the responses to KLH in the presence of CyaA were significantly greater than those in mice immunized with KLH alone (Fig. 4.6B). Comparable with the data after a single immunization, IgG1 was the dominant subclass of the antibody response. These data clearly demonstrate that CyaA acts an adjuvant for antibody, as well as T cell responses *in vivo*.

To examine the possible contribution of the small amount of LPS in the preparation of CyaA (200 pg LPS / μg CyaA) to this adjuvanticity, C3H/HeN (wildtype) and C3H/HeJ (TLR4-hyporesponsive) mice were co-injected with KLH alone or with CyaA. KLH-specific antibody responses were assessed. Consistent with the results from BALB/c mice, significantly higher levels of KLH-specific IgG1 were detected in the serum of C3H/HeN mice immunised with KLH and CyaA compared with mice immunised with KLH alone (Fig. 4.7A). IgG2a levels were not significantly different between the two groups. This pattern of antigen-specific IgG1 polarization was again observed after a second immunization (Fig. 4.7B). Similarly, C3H/HeJ mice immunised with KLH and CyaA demonstrated a significant increase in KLH-specific IgG1 antibodies compared

with those that were immunized with KLH alone (Fig. 4.7A) with no difference between KLH-specific IgG2a antibodies between the two groups. Interestingly, this increase in KLH-specific IgG1 was less than that observed in the wildtype C3H/HeN mice. However, after a second immunization the amount of IgG1 produced by C3H/HeN and C3H/HeJ mice immunised with KLH and CyaA was similar (Fig. 4.7B). While there may be a role for signalling through TLR4 in the adjuvanticity of CyaA *in vivo*, these results clearly demonstrate that CyaA acts as an adjuvant for co-administered antigens, polarising the antibody production to the IgG1 subtype, and that this is not dependant on LPS.

4.2.3 T cell response driven by CyaA is not detected in TLR-4-defective mice.

The antigen specific T cell response in C3H/HeN (wildtype) and C3H/HeJ (TLR-4-defective) was examined in order to further investigate the role of TLR-4 signalling in the adjuvanticity of CyaA *in vivo*. Groups of C3H/HeN and C3H/HeJ mice were immunised in the hind footpad with KLH (5 μg) and CyaA (1 μg) or KLH alone. Seven days after immunisation, mice were sacrificed and lymph node cells were restimulated with antigen (2-50 μg/ml) *in vitro* and cytokine concentrations in the supernatants were determined by ELISA 3 days later. Cells from both C3H/HeN and C3H/HeJ mice responded to the polyclonal stimulus of PMA and anti-CD3, producing IFN-γ, IL-4, IL-5 and IL-10 (Fig. 4.8). Similar to BALB/c mice, immunisation of C3H/HeN mice with KLH alone generated a weak cellular immune response with production of low levels of IL-10 and IFN-γ (Fig. 4.8A). However, cells restimulated with antigen from C3H/HeN mice immunised with KLH and CyaA produced much higher concentrations of IL-10 with low levels of IFN-γ. In contrast to cells from BALB/c mice immunised with KLH and CyaA (Figs. 4.1 and 4.3), cells from C3H/HeN mice did not produce detectable levels of IL-4 or IL-5. Immunisation of C3H/HeJ mice with KLH or KLH with CyaA did not result in the production of IFN-γ, IL-4, IL-5 or IL-10 (Fig. 4.8B). However, these cells did have the capacity to produce these cytokines, as these were detected after stimulation with PMA and anti-CD3. The fact that no antigen-specific T cell cytokines were detected from TLR-4-defective mice immunised with KLH and CyaA suggests that there is a role for LPS and TLR-4 signalling in the adjuvanticity of

CyaA. However, given that the recall response in C3H/HeN (wildtype) mice differs from that in BALB/c mice and that antibody production in C3H/HeJ mice is only marginally reduced shortly after immunisation compared to C3H/HeN (wildtype) it may also indicate a strain specific or temporal difference in the detection of the T cell response promoted by CyaA in BALB/c, C3H/HeN and C3H/HeJ mice.

4.2.4 CyaA modulates cytokine production from innate cells.

Cells of the innate immune system, including DC and macrophages direct the adaptive immune response by presenting antigens and secreting regulatory cytokines. To investigate the effect of CyaA on these cells, J774 macrophages and immature bone marrow-derived DC were incubated with CyaA (1 μg/ml), *E. coli* LPS (1-1000 ng/ml) or CyaA and LPS Since the CyaA protein is associated with LPS, which was reduced but not completely eliminated during purification, it was important to determine the role, if any, of this LPS in the immunomodulatory effects of CyaA. Therefore cells were also stimulated with CyaA in the presence of polymyxin B. Supernatants were collected 2, 4 and 28 h after stimulation and assayed for cytokines. The purified CyaA, that included residual LPS (220 pg/ml), stimulated low levels of IL-6, IL-10 and TNF-α production from J774 cells (Fig. 4.9) and low levels of IL-6 and TNF-α (but no IL-10) secretion from DC (Fig. 4.10). This cytokine production was abrogated in the presence of polymyxin B. However, stimulation with LPS alone at the dose present in the CyaA preparation (220 pg/ml) did not induce production of these cytokines (Figs 4.9 and 4.10). Furthermore, polymyxin B alone did not affect cytokine production by macrophages or DC (data not shown). These data suggest that CyaA induces cytokine production by innate cells only in the presence of LPS.

The effect of CyaA on cytokine production in response to increasing concentrations of LPS was also examined. CyaA synergised with LPS in promoting IL-6 and IL-10 production from macrophages and DC. IL-10 production from macrophages stimulated with CyaA and LPS (1-1000 ng/ml) was significantly higher than that of macrophages stimulated with the corresponding dose of LPS alone at all time points examined (Fig. 4.9). IL-10 could not be detected in supernatants of DC 4 h after stimulation with LPS

(1-1000 ng/ml) alone, whereas significant levels of IL-10 were produced following addition of CyaA (Fig. 4.10). LPS-induced IL-6 production by macrophages and DC was also significantly enhanced by the addition of CyaA, but this was only observed at early time points.. Interestingly, the synergistic effects of CyaA upon LPS induced IL-6 were only detectable at the early time points of 2 and 4 h. In contrast to the positive effect on IL-6 and IL-10 production, CyaA suppressed TNF-α secretion from macrophages and DC and IL-12p70 production from DC. These inhibitory effects were observed at the three time points examined and over a range of concentrations of LPS. These data demonstrate that CyaA alone has little enhancing effect on cytokine production by cells of the innate immune system, but can synergise with a TLR ligand, even at very low concentrations, in promoting IL-10 production, while inhibiting TNF-α and IL-12 production.

4.2.5 Effect of CyaA on maturation of DC.

Several pathogen-derived molecules that bind to TLRs, induce maturation of immature DC (Akira, 2003, de Jong *et al.*, 2002, Hemmi *et al.*, 2000), thereby enhancing their capacity to activate naïve T cells. Therefore, the ability of CyaA to stimulate the maturation of DC and/or to modulate LPS-induced maturation was examined. Immature DC were stimulated with CyaA, LPS or LPS with CyaA and the expression of surface markers associated with maturation was examined by immunoflourescence analysis 24 h later. As expected, LPS (1 μg/ml), enhanced surface expression of CD80, CD86, MHC class II, CD40 and ICAM-I (Fig. 4.11). Stimulation of immature DC with CyaA (in the presence of polymyxin B) also resulted in upregulation of surface expression of CD80 and MHC class II, and to a lesser extent CD86. In contrast, expression of CD40 and ICAM-I was downregulated following incubation with CyaA. Furthermore, CyaA inhibited LPS-induced upregulation of CD40, ICAM-I and CD86. In contrast, treatment of cells with LPS at the level present in the CyaA preparation (220 pg/ml) had no effect on DC surface marker expression (data not shown). These findings demonstrate that CyaA treatment results in partial maturation of the DC, upregulating CD80 and MHC-II, but inhibiting CD40 and ICAM-1, a phenotype distinct from mature DC that drive

the differentiation of Th1 cells, but similar to those that promote the induction of Tr1 cells (Lavelle *et al.*, 2003, McGuirk *et al.*, 2002, Mills, 2001).

4.2.6 Effect of CyaA on DC from TLR-4 defective mice.

To further address the role of LPS in the mechanism of action of CyaA as an adjuvant and immunomodulator, innate cytokine and chemokine production and maturation of DC from TLR4-defective mice was examined. DC from TLR-4-defective C3H/HeJ and immunocompetent C3H/HeN mice were treated with CyaA, LPS, LPS and CyaA or CyaA and polymyxin B and supernatants recovered after 4 h. CyaA (that included 220 pg/ml residual LPS) stimulated IL-10 production by DC from C3H/HeN, but not from C3H/HeJ mice (Fig. 4.12). Furthermore, IL-10 production by DC from C3H/HeN mice was inhibited by polymyxin B. However, LPS alone at the concentration present in the CyaA preparation (220 pg/μg) did not induce IL-10, IL-12p70, TNF-α or MIP-1α production by DC from C3H/HeN mice. Addition of a higher dose of exogenous LPS (10 ng/ml) did not induce significant IL-10 at the 4 h time point, but synergized with CyaA in promoting IL-10 production. Furthermore, CyaA suppressed LPS induced IL-12p70, TNF-α and MIP-1α production by DC from C3H/HeN mice (Fig. 4.12), but had no effect on cytokine production by DC from C3H/HeJ mice (Fig. 4.12). In contrast, CpG, a TLR9 ligand, activated cytokine production by C3H/HeN and C3H/HeJ DC in a similar fashion (Fig. 4.12). These data provide further evidence that CyaA, although having no direct effect on cytokine production by DC, modulates LPS-induced responses, synergising with LPS to induce IL-10 and suppressing LPS induced production of IL-12p70, TNF-α and MIP-1α.

The effects of CyaA on maturation of DC from C3H/HeN and C3H/HeJ mice was also examined. As shown for BALB/c mice, CyaA induced maturation of DC from C3H/HeN mice, specifically CyaA enhanced expression of CD80, CD86, MHC class II, CD40 and ICAM-I (Fig 4.13A). In the presence of polymyxin B these effects were diminished, in particular CD40, ICAM-1, and MHC class II, which were expressed at lower levels than those seen on medium-treated control DC. LPS-induced expression of CD86, CD40 and ICAM-I was also inhibited by CyaA, though not to the same extent as that observed in DC from BALB/c mice. In contrast to the modulatory effects in

C3H/HeN mice, LPS, CyaA or LPS with CyaA had no enhancing effects on CD86, MHC class II, CD40 or ICAM-I but did have a modest, but reproducible, effect on CD80 on DC from C3H/HeJ mice (Fig. 4.13B). However CyaA marginally reduced CD40 and ICAM-I expression on DC from C3H/HeJ mice in the presence of polymyxin B. These findings suggest that upregulation of maturation markers on DC by CyaA is dependant on a second signal, such as LPS even at very low doses, but that inhibition of endogenous expression of CD40 and ICAM-I can occur in the absence of LPS.

4.3 Discussion

This study demonstrated that CyaA can selectively promote the induction of Th2 and Tr1 responses to co-administered antigens *in vivo* through its modulatory effect on DC, but that the effects on DC were facilitated by on a second signal provided by LPS signalling through TLR4. It was previously reported that CyaA could enhance antibody responses to co-injected antigen (Hormozi *et al.*, 1999) and when used as a vector for viral peptide antigens it resulted in the stimulation of $CD8^+$ CTL and Th1 responses against the viral peptide (Dadaglio *et al.*, 2000, Fayolle *et al.*, 1996). The results here show that CyaA enhanced IgG1 antibodies and IL-5 and IL-10 production by antigen-stimulated lymph node cells, but had very little effect on IFN-γ production. Furthermore, antigen-specific $CD4^+$ T cell clones generated from mice immunized with KLH in the presence of CyaA secreted IL-5 and IL-10 or IL-4, IL-5 and IL-10, suggesting that it selectively promoted the generation of Th2 and Tr1 cells *in vivo*.

A number of bacterial toxins and virulence factors have been shown to modulate immune responses as a strategy to evade protective immunity and prolong their survival in the host (Mills, 2001). Moreover, certain of these molecules, including CT, *E. coli* heat labile enterotoxin and LPS can also act as adjuvants enhancing immune responses to co-administered antigens (Ryan *et al.*, 2001). The adjuvant properties of CyaA may be related to its ability to bind to CD11b/CD18 on innate cells (Guermonprez *et al.*, 2002, Guermonprez *et al.*, 2001). CyaA binding to CD11b/CD18,

expressed on DC, macrophages and other innate cells, has been exploited as a means of targeting foreign peptide antigens to MHC class I and class II processing pathways (Fayolle *et al.*, 1999, Loucka *et al.*, 2002). FHA from *B. pertussis* also binds to CD11b/CD18 (as well as to CD47/CD61) and it has been demonstrated that FHA can stimulate IL-10 production from DC and macrophages and activate maturation of DC into a phenotype that promotes the differentiation of naïve T cells into Tr1 cells (McGuirk *et al.*, 2002). Furthermore CT has been shown to promote the activation of Tr1 cells, as well as Th2 cells, by modulating DC activation (Lavelle *et al.*, 2003).

The induction of antigen-specific Tr cells by pathogens or pathogen-derived molecules and their role in infectious diseases is only beginning to be understood. It appears that these cells are generated during chronic or persistent infection, either as an evasion strategy by the pathogen to inhibit protective immune responses or as a host protective mechanism to limit collateral damage mediated by inflammatory Th1 or Th2 cells (McGuirk and Mills, 2002). Tr cells also have an important role in preventing autoimmune diseases and allergy mediated by Th1 cells and Th2 cells respectively, therefore their induction *in vivo* may be a useful therapeutic strategy for immune mediated diseases. Current evidence suggests that IL-10, and possibly IL-4 and IFN-α, may play a role in promoting the induction of Tr1 cells from naive T cells *in vivo* (McGuirk and Mills, 2002, Mendel and Shevach, 2002). In contrast, innate IL-12 and IL-27 enhance Th1 responses, while IL-4 and IL-6 have been implicated in directing the induction of Th2 cells (Moser and Murphy, 2000, Rincon *et al.*, 1997, Robinson and O'Garra, 2002). It has also been suggested that IL-6 induced by microbial products through the TLR pathway may inhibit DC activation of $CD4^+CD25^+$ natural Tr cells (Pasare and Medzhitov, 2003). The results of the present study support a role for macrophage and DC derived IL-10 and IL-6 directing the induction of Tr1 and Th2 cells. CyaA, in synergy with low doses of LPS, enhances IL-6 and IL-10 production from DC, but inhibits LPS-induced IL-12 and TNF-α production.

In addition to regulatory cytokine secretion, the maturation status of the DC also influences T cell subtype induction. Many pathogen-derived molecules that bind to TLRs promote the maturation of DC that direct the induction of Th1 cells. Emerging evidence indicates that DC which promote activation of Tr cells are not immature or

fully mature but have an intermediate phenotype (Lavelle *et al.*, 2003, McGuirk and Mills, 2002). This study found that CyaA enhanced CD80, but reduced CD40 and ICAM-I expression on murine DC when LPS effects were abolished from our CyaA preparation with polymyxin B. It had previously been reported that commercially available *E. coli*-expressed CyaA enhances CD80, CD86, CD83 and MHC class II on human DC (Bagley *et al.*, 2002b). We failed to detect significant enhancement of CD86 on murine DC and in fact LPS-induced CD86 was suppressed by incubation with CyaA, as was LPS-induced CD40 and ICAM-I.

Studies on immunomodulation with purified native or recombinant pathogen-derived molecules are complicated by the possible contamination with low levels of other molecules, which may also have immunomodulatory activities. This is a problem with molecules expressed in *E. coli* or purified from Gram-negative bacteria, since LPS is often difficult to remove or dissociate from protein molecules, especially RTX toxins (Czuprynski and Welch, 1995, Li and Clinkenbeard, 1999). The primary objective of our studies was to examine the immunomodulatory effects of CyaA and to try to distinguish these effects from those generated with the protein in the presence of LPS. The work here, with a recombinant *E. coli* expressed protein has important parallels for any future immunotherapeutic applications of CyaA, which are more likely to employ recombinant CyaA molecules produced under good-manufacturing-practice (GMP) conditions in the absence of LPS and other contaminants. However, during *B. pertussis* infection host cells will respond to CyaA in the context of high concentrations of LPS. Furthermore, CyaA, in addition to being secreted by *B. pertussis,* can be delivered to the host epithelial cells upon contact with *B. pertussis.* Although it has been considered that *B. pertussis* are primarily localized at the epithelial surface, there is also evidence that it can infect human macrophages (Mills, 2001). Furthermore, immature myeloid DC, which express CD11b and other pathogen recognition receptors, are present in respiratory airways (Piggott *et al.*, 2005) and will be activated by different *B. pertussis* virulence factors, including CyaA and LPS, before migrating to the lymph node, where they direct the induction of naïve T cells. Indeed *B. pertussis*-specific Tr1 and Th1 cells have been detected in lymph nodes and spleen from mice infected with *B. pertussis*

(Higgins *et al.*, 2003, McGuirk *et al.*, 2002). Circulating IFN-γ-secreting Th1 cells were also detected in children infected with *B. pertussis* (Ryan *et al.*, 1997).

Interestingly, while the data here shows that CyaA promotes the production of antigen specific IgG1 in the absence of TLR-4 signalling it does suggest that this production is less than optimal but the responses in C3H/HeJ mice can be restored to that of C3H/HeN mice by a second immunisation. Surprisingly, no adaptive cytokine production was detected in response to antigen restimulation of lymph node cells from TLR-4-hyporesponsive mice immunised with KLH in the presence of CyaA. This suggests a role for LPS and TLR signalling in the adjuvanticity of CyaA. However, there were differences between the recall response in BALB/c and C3H/HeN (wildtype) mice immunised with KLH and CyaA, therefore the lack of an antigen specific recall response in C3H/HeJ (TLR-4-defective) mice may also represent a difference between the strains of mice in the strength of their T cell response or a temporal difference in detection of the response.

The assumption that low levels of contaminating LPS do not contribute to immunological activity of a protein because the same dose of LPS alone has no demonstrable effect or that denaturing the protein abrogates the activity may not take into account possible co-operative or synergistic effects. Our findings demonstrate that CyaA does have adjuvant and immunomodulatory properties, but that this effect may be partly dependant on additional signalling induced by LPS through TLR4. Purified CyaA with low levels of contaminating LPS (220 pg/μg protein) enhanced certain aspects of DC maturation, including upregulation of CD80 and MHC class II expression and stimulation of low levels of IL-6, IL-10 and TNF-α production from DC and macrophages. Many of the activities of the purified CyaA preparation, including induction of TNF-α, IL-10 and IL-6 production and enhancement of CD80 and MHC class II expression on DC were abrogated following the addition of polymyxin B or through use of TLR4-defective mice. This suggests a TLR-4 dependant mechanism in the modulation of DC activation and maturation by CyaA. TLR signalling may be required as a second signal with CyaA to fully activate these important cells of the innate immunse system to prime the T cell response, which could explain the lack of cytokine production detected from T cells from C3H/HeJ (TLR-4-

defective) mice immunised with KLH and CyaA. However LPS at a concentration equivalent to that present in our CyaA preparation (220 pg/μg CyaA protein) did not itself induce innate cytokine production or DC maturation, but did significantly augment the effect of the CyaA. Furthermore, addition of increasing amounts of exogenous LPS (1-1000 ng/ml) significantly augmented IL-10 and IL-6 production by DC and macrophages. Synergistic effects between CyaA and LPS were particularly evident at early time points after cell stimulation, when the same dose of LPS alone had little effect.

The concept that two signals are required for immune activation is fully recognized for T cells, but there is growing evidence for this phenomenon for cells of the innate immune system. PT has been shown to synergise with LPS to promote IL-12 production from DC (de Jong *et al.*, 2002). Furthermore, CT synergises with LPS, or other TLR ligands, including CpG motifs or poly(I:C), to stimulate IL-10 production from DC and macrophages (Lavelle *et al.*, 2003). Elevation of intracellular cAMP and possibly activation of protein kinase A may have been implicated in the immunomodulatory effects of CT and of CyaA (Bagley *et al.*, 2002b), suggesting that cAMP-dependant signalling pathways are involved in innate cell activation by CyaA. However the present study, combined with previous reports on synergy between CT and different TLR ligands from this laboratory (Lavelle *et al.*, 2003) and the demonstration that innate IL-10 production and induction of Tr1 cells following *B. pertussis* infection is compromised in TLR4-defective mice (Higgins *et al.*, 2003), suggest that signalling pathways leading to DC maturation and IL-10 and IL-6 production following CyaA recognition of CD11b/CD18 may be dependent on co-operative activation through a TLR.

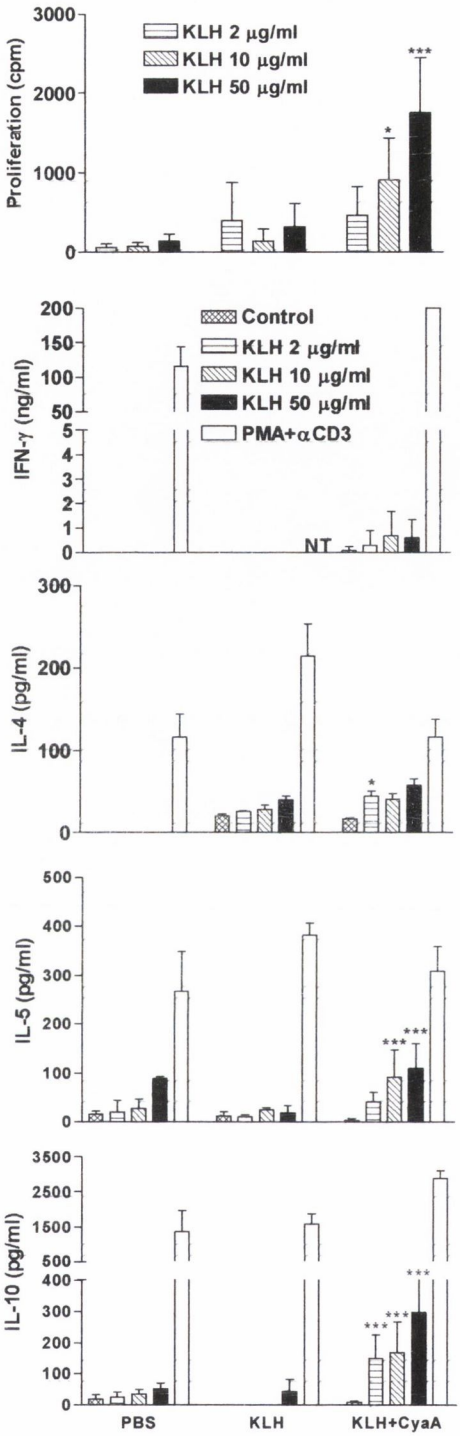


Figure 4.1: CyaA induces Th2/Tr1 responses to a co-administered antigen. Female BALB/c mice were immunized s.c. in the hind footpad with PBS, KLH (5 µg) alone or with CyaA (1 µg). After 7 days mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 µg/ml), PMA and anti-CD3 or medium only. After 3 days supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by [^{3}H]thymidine incorporation. Results represent means (error bars, SD) of five mice per group and are representative of three experiments. NT, not tested, *, $P < 0.05$; ***, $P < 0.001$ KLH versus KLH + CyaA.

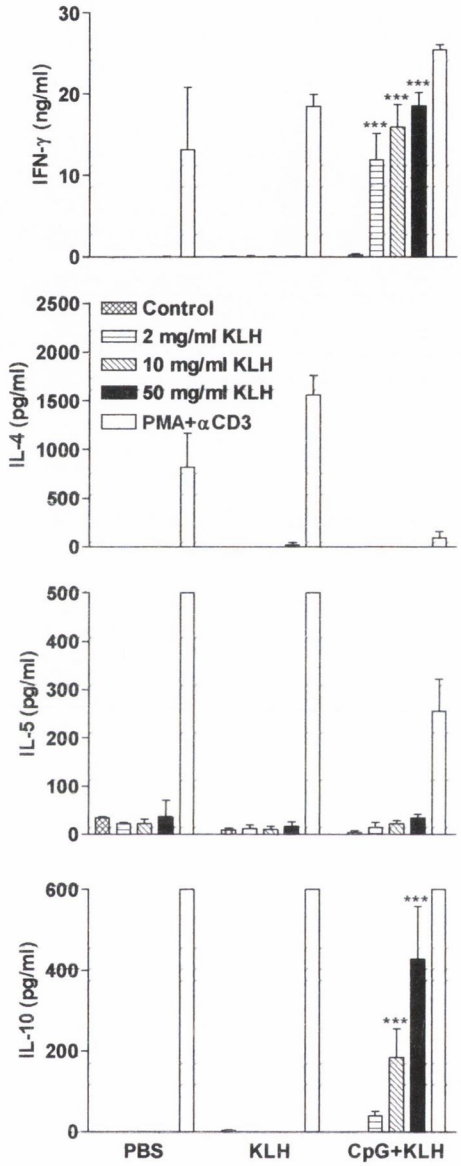


Figure 4.2 CpG-ODN induces antigen-specific Th1 cell production of IFN-γ and IL-10. Female BALB/c mice were immunized s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CpG-ODN (25 μg). After 7 days mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 μg/ml), PMA and anti-CD3 or medium only. After 3 days supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by [^{3}H]thymidine incorporation. Results represent means (error bars, SD) of five mice per group and are representative of two experiments. ***P<0.001 versus KLH alone.

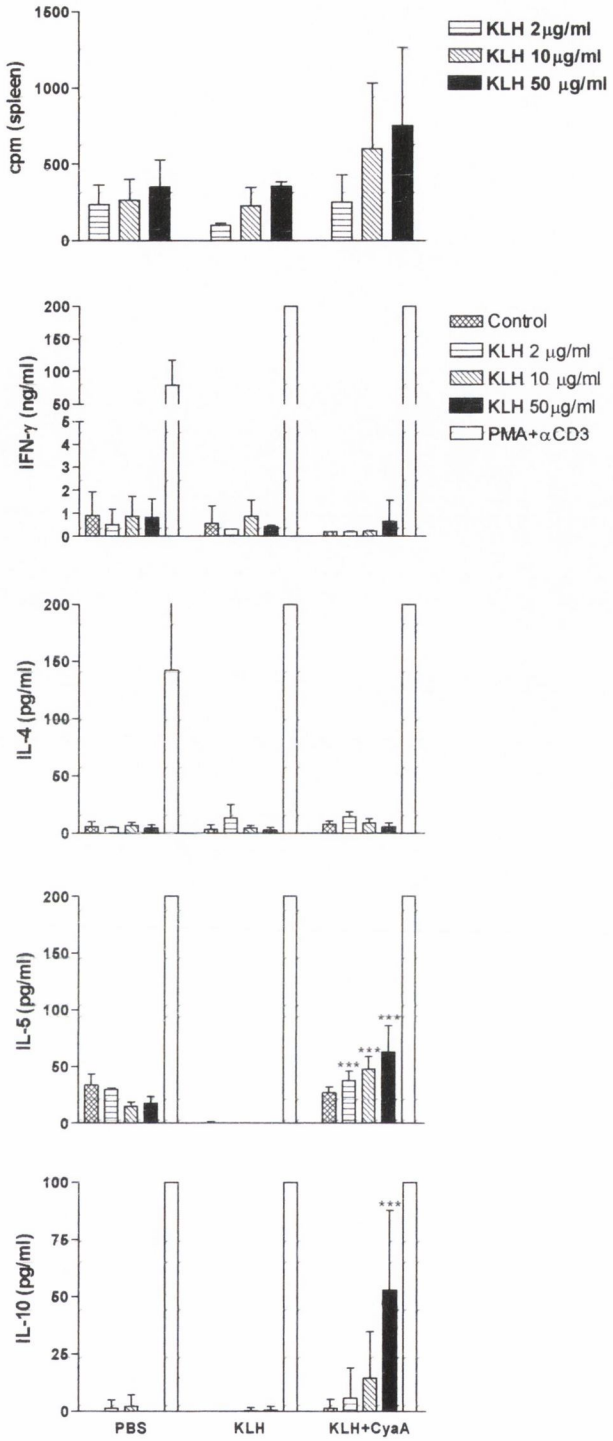


Figure 4.3 CyaA induces Th2/Tr1 responses after a secondary immunisation with antigen and CyaA. Female BALB/c mice were immunized s.c. in the hind footpad with PBS, KLH (5 µg) alone or with CyaA (1 µg). After 21 days mice were boosted with PBS, KLH (5 µg) alone or with CyaA (1 µg) respectively. After a further 7 days mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 µg/ml), PMA and anti-CD3 or medium only. After 3 days supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by [^{3}H]thymidine incorporation. Results represent means (error bars, SD) of five mice per group and are representative of two experiments. ***P<0.001 versus KLH alone.

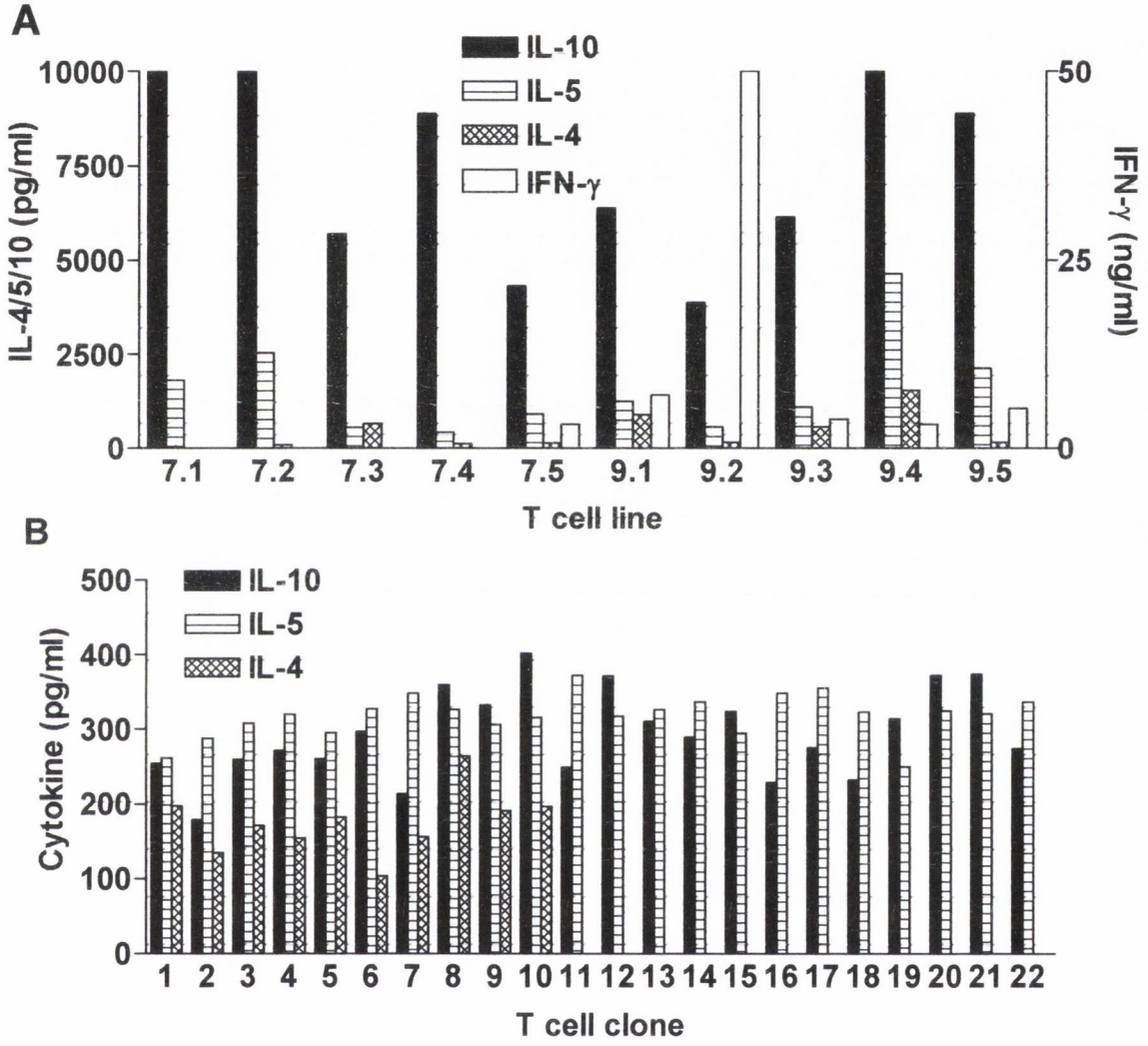


Figure 4.4: KLH-specific Th2 and Tr1 cell lines and clones generated from mice immunized with KLH in the presence of CyaA. (A) $CD4^+$ T cell lines were generated from lymph nodes of 10 individual mice immunized with KLH and CyaA. (B) T cell line 7.2 was cloned by limiting dilution. T cell lines or clones were stimulated with KLH (50 μg/ml) in the presence of autologous APC and cytokine concentrations tested in the supernatants after 3 days. Results are cytokine profiles for lines or clones cultured in individual wells of 96 or 24 well plates and are representative of 3 different assays. IFN-γ was at background levels in each of the T cell clones.

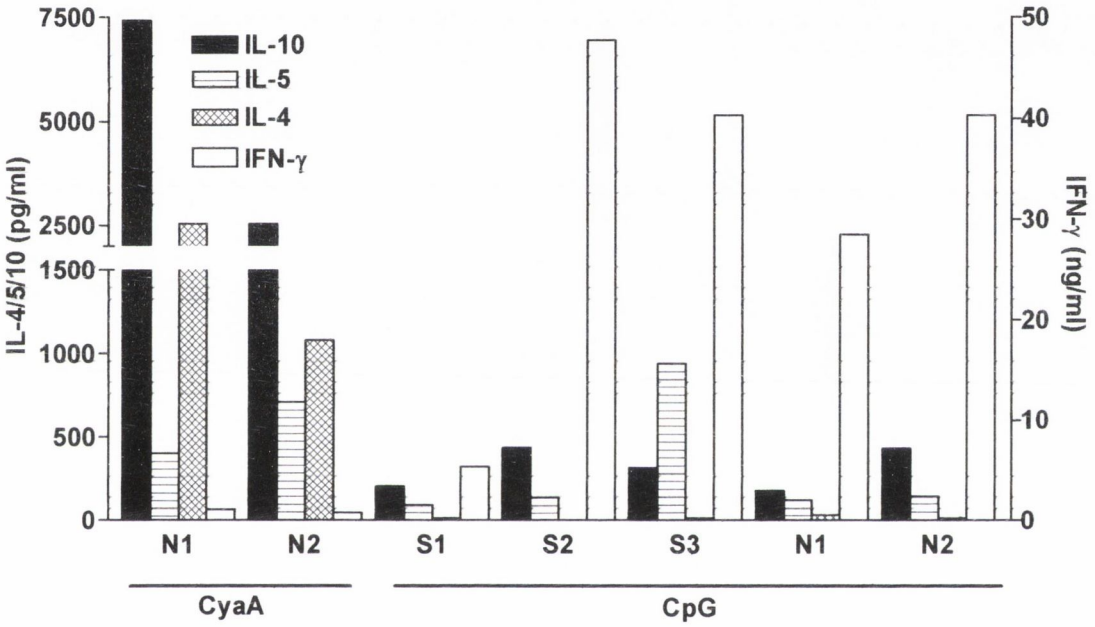


Figure 4.5: KLH-specific Th2 and Tr1 cell lines generated from mice immunized with KLH in the presence of CyaA and Th1 cell lines generated from mice immunized with KLH in the presence of CpG-ODN. $CD4^+$ T cell lines were generated from lymph nodes (N) and spleens (S) of individual mice immunized with KLH (5 μg) and CyaA (1 μg) or KLH and CpG-ODN (25 μg). T cell lines were stimulated with KLH (50 μg/ml) in the presence of autologous APC and cytokine concentrations tested in the supernatants after 3 days. Results are cytokine profiles for lines cultured in individual wells of 24 well plates and are representative of 2 different assays.

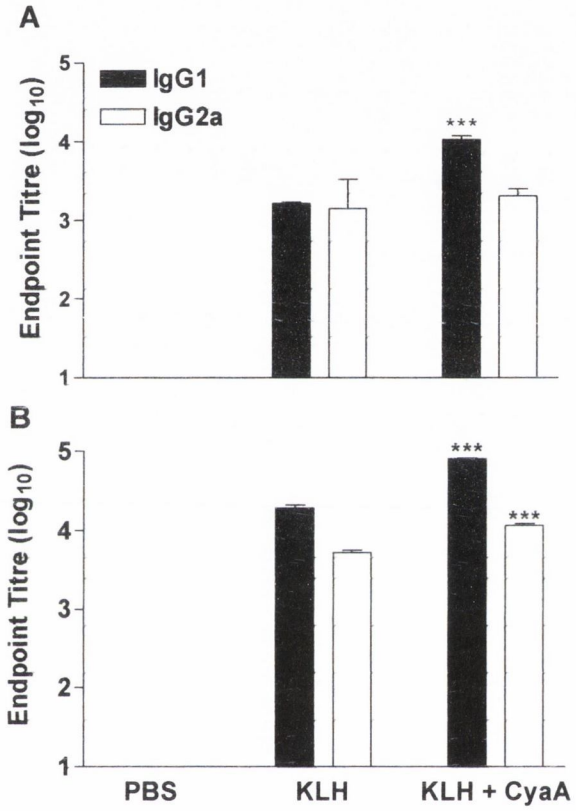


Figure 4.6: CyaA enhances IgG1 antibodies to co-administered antigens in vivo. Mice were immunized s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg) and boosted 21 days later. Serum samples taken 7 days after one (A) or two (B) immunization(s) and KLH-specific IgG, IgG1 and IgG2a determined by ELISA. Results are mean (+ SD) titres for 5 mice per group and are representative of 2 experiments. ***, $P < 0.001$ KLH versus KLH + CyaA.

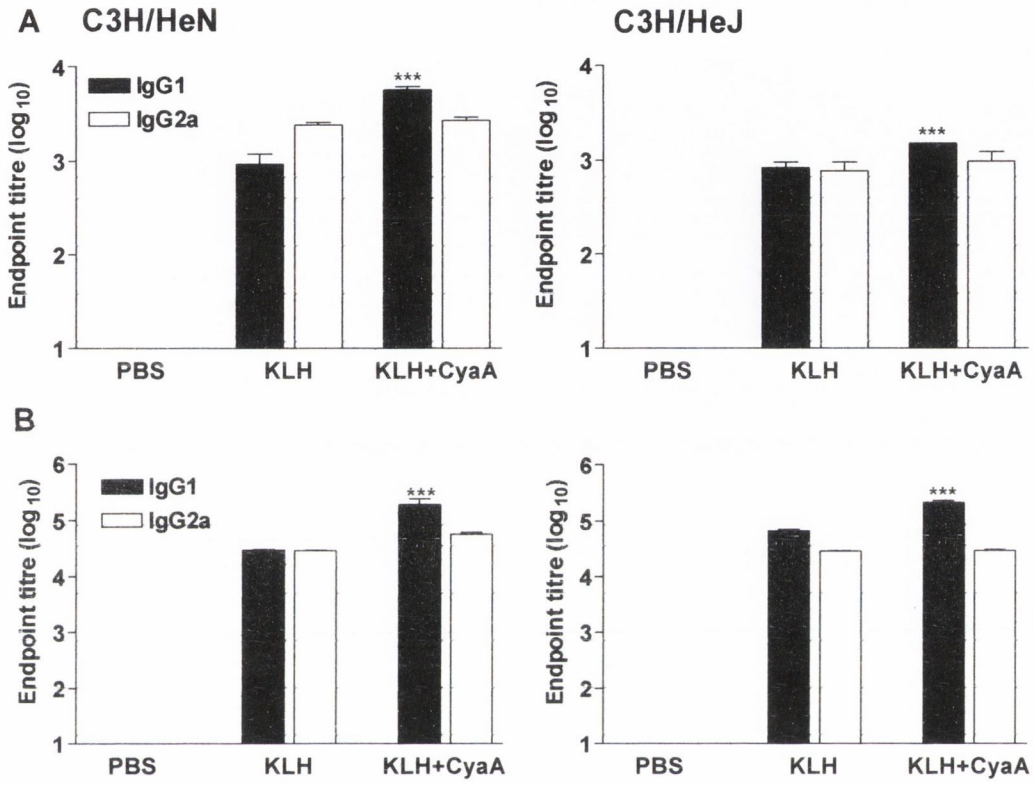


Figure 4.7: CyaA enhances IgG1 antibodies to co-administered antigens in vivo in TLR-4-defective mice. C3H/HeN (wildtype) and C3H/HeJ (TLR-4-defective) mice were immunized s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg) and boosted 21 days later. Serum samples taken 7 days after the first (A) or second (B) immunization and KLH-specific IgG1 and IgG2a determined by ELISA. Results are mean (+ SD) titres for 5 mice per group and are representative of 2 experiments. ***, $P < 0.001$ KLH versus KLH + CyaA.

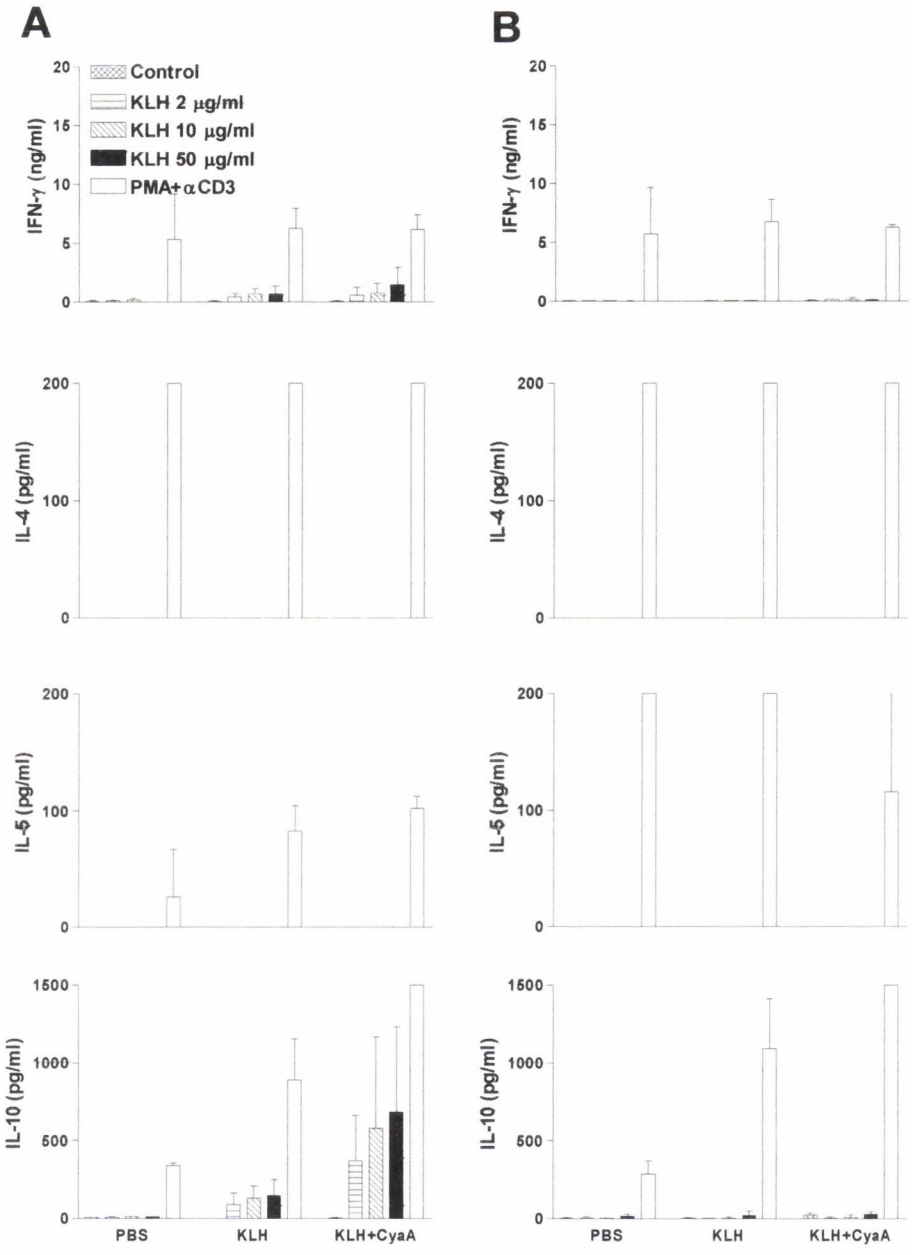


Figure 4.8 CyaA induces Th2/Tr1 responses in C3H/HeN mice after immunisation with antigen and CyaA, while these responses are reduced in C3H/HeJ mice. Female C3H/HeN (A) and C3H/HeJ (B) mice were immunized s.c. in the hind footpad with PBS, KLH (5 µg) alone or with CyaA (1 µg). After 7 days mice mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 µg/ml), PMA and anti-CD3 or medium only. After 3 days supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by [^{3}H]thymidine incorporation. Results represent means (error bars, SD) of five mice per group and are representative of two experiments.

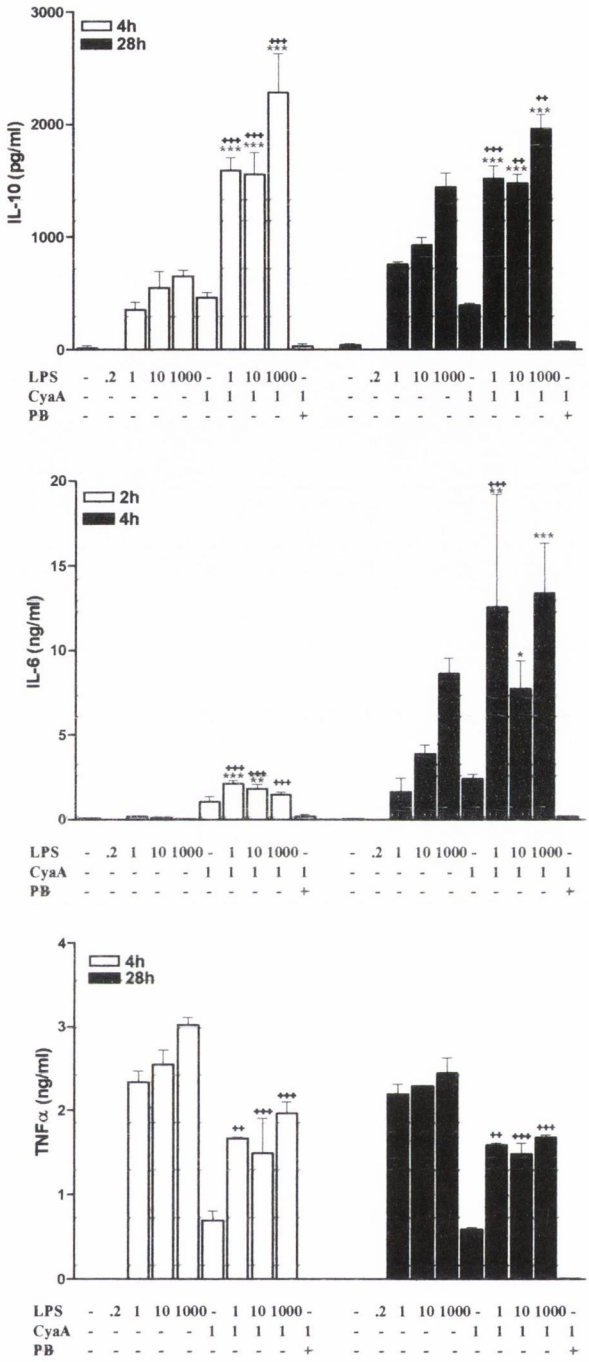
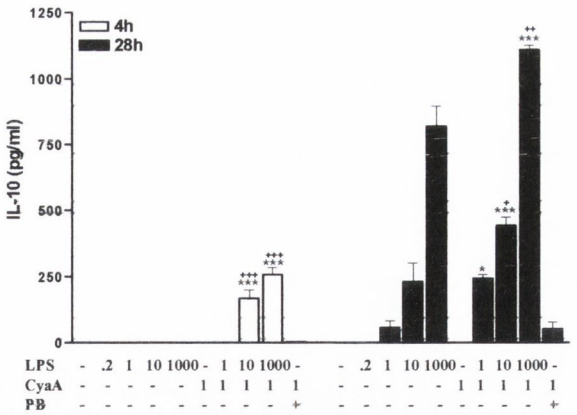


Figure 4.9: CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses pro-inflammatory cytokines by macrophages. J774 macrophages ($1x10^6$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml), in presence or absence of polymyxin B (PB; 10 μg/ml). Supernatants were collected at the indicated times and were tested for IL-10, IL-6 and TNF-α by immunoassay. Results are means (+ SD) of triplicate assays and are representative of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus CyaA; ++, $P < 0.01$, +++, $P < 0.001$; versus LPS alone at the same concentration.

Figue 4.10: CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses LPS-induced pro-inflammatory cytokines from DC. Murine bone marrow-derived immature DC ($1x10^6$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml) in presence or absence of polymyxin-B (PB; 10 μg/ml). Supernatants were collected at the indicated times and tested for IL-10, IL-6, TNF-α and IL-12p70 by immunoassay. Results are means (error bars, SD) of triplicate assays and are representative of three experiments. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$, versus LPS alone at the same concentration, **, $P < 0.01$; ***, $P < 0.001$ versus CyaA.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28h
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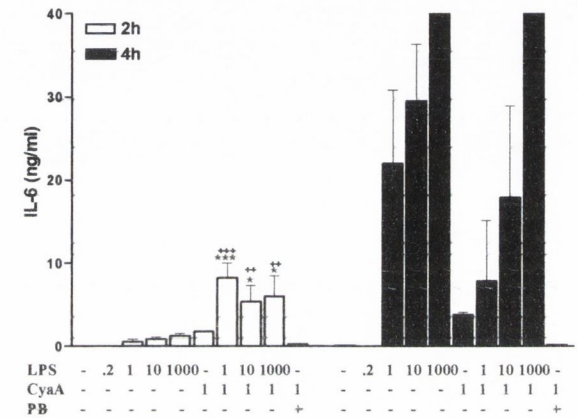
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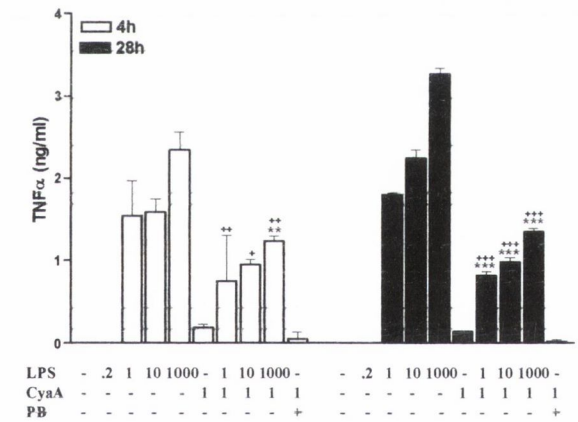
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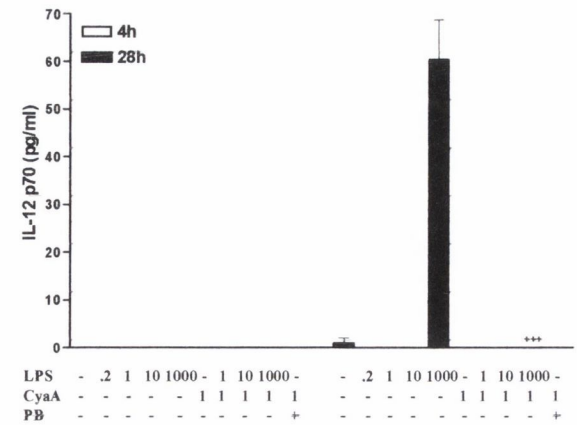
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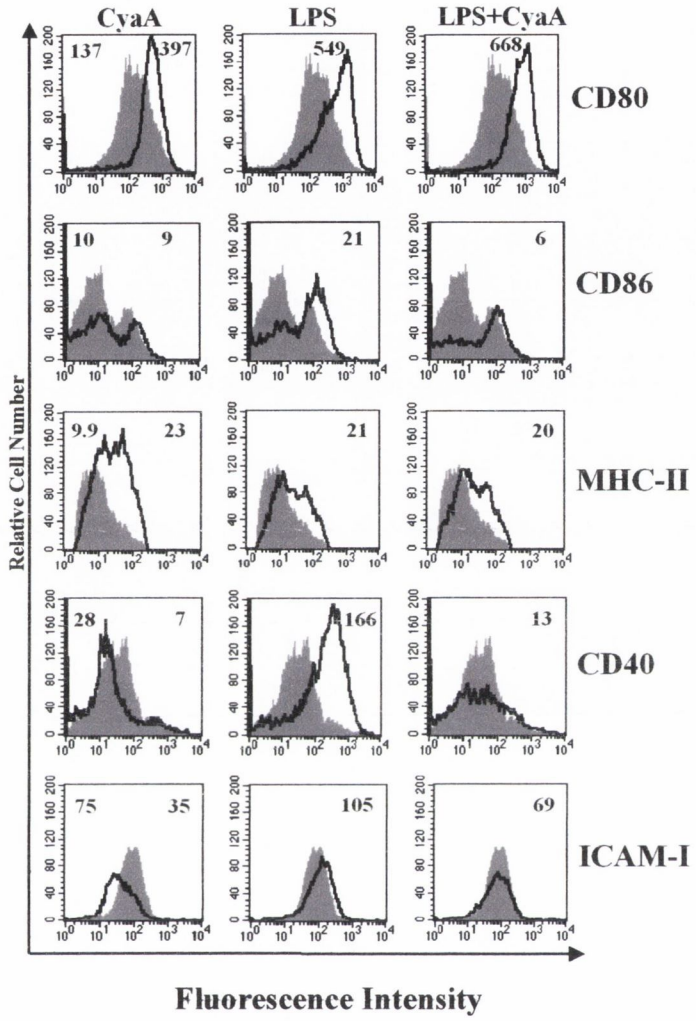

Figure 4.11: CyaA enhances CD80, CD86 and MHC-II, but inhibits CD40 and ICAM-I expression on DC. DC were stimulated with CyaA (1 μg/ml) in the presence of polymyxin B (10 μg/ml), LPS (1 μg/ml), CyaA + LPS or medium only. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched controls. Results of immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) DC. Profiles are shown for a single experiment and are representative of 2 separate experiments.

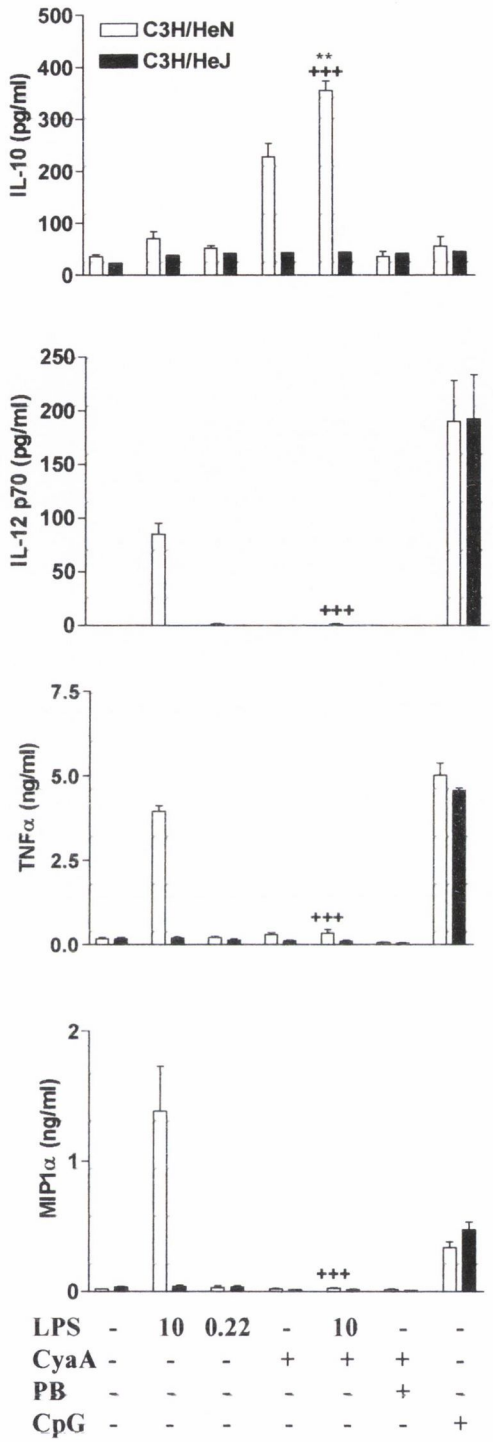

Figure 4.12: CyaA-induced cytokine and chemokine production is altered in TLR4-defective mice. Bone marrow derived DC from C3H/HeN or C3H/HeJ mice ($1x10^6$/ml) were cultured with the indicated concentrations of LPS (0-10 ng/ml), CyaA (1 μg/ml), in the presence or absence of polymyxin B (PB; 10 μg/ml). Supernatants were collected and tested by immunoassay for IL-10 and MIP1-α (4 h) and IL-12p70 and TNF-α (24 h). Results are means (error bars, SD) of triplicate assays and are representative of three experiments. **, $P < 0.01$ versus CyaA; +++, $P < 0.001$ versus LPS alone at the same concentration.

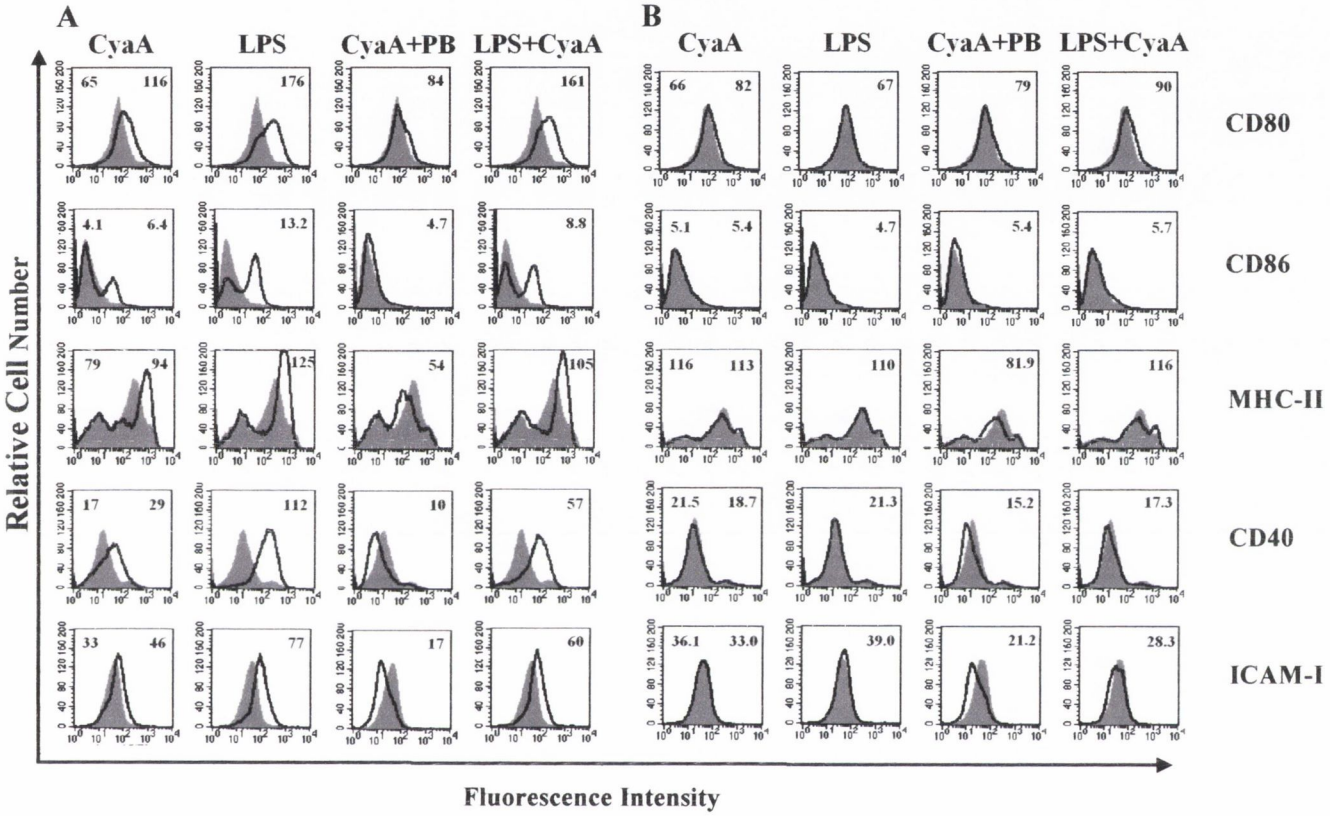

Figure 4.13: CyaA-induced DC activation is altered in TLR4-defective mice. Bone marrow derived DC from C3H/HeN (A) or C3H/HeJ (B) mice ($1x10^6$/ml) were cultured with CyaA (1 μg/ml) either alone or with polymyxin B (PB; 10 μg/ml) or LPS (10 ng/ml) or with medium. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched control antibodies. Immunofluorescence analysis are shown for treated (black line) compared with untreated (grey histogram) DC. The numbers on the right of each histogram refer to the mean fluorescence intensity of the treated cells for the relevant fluorescent antibody, the value for cells treated with medium only is shown on the left of the first histogram in each case. Profiles are shown for a single experiment and are representative of 3 experiments.

CHAPTER 5

DISTINCT ROLES FOR ACYLATION AND ENZYMATIC ACTIVITY OF CYAA IN IMMUNOMODULATION AND CELL DEATH

5.1 Introduction

Bordetella pertussis, the causative agent of whooping cough, and *Bordetella bronchiseptica* which causes chronic respiratory disease in a variety of mammals, both express adenylate cyclase toxin (CyaA) (Goyard *et al.*, 1993). This toxin is encoded by the *cyaA* gene and is an essential virulence factor for both *Bordetella* species. CyaA specifically binds to the $\alpha_M\beta_2$ integrin (CD11b/CD18) on the surface of macrophages and DC (El-Azami-El-Idrissi *et al.*, 2003, Guermonprez *et al.*, 2001). Upon entry into target eukaryotic cells, CyaA is activated by binding calmodulin and converts ATP into cAMP, generating supraphysiological concentrations of this powerful signalling molecule (Confer and Eaton, 1982). Treatment of neutrophils with CyaA leads to inhibition of chemotaxis, phagocytosis and superoxide generation (Bassinet *et al.*, 2000, Confer and Eaton, 1982). CyaA has also been shown to induce apoptosis in macrophages (Khelef and Guiso, 1995, Khelef *et al.*, 1993).

The results presented in chapter 4, along with the work from other laboratories, have shown that CyaA of *B. pertussis* is an effective vaccine adjuvant *in vivo*, enhancing antibody responses to co-administered antigens (Dadaglio *et al.*, 2000, Hormozi *et al.*, 1999, Macdonald-Fyall *et al.*, 2004). The data in chapter 4 also demonstrated that CyaA promotes Th2 and type 1 regulatory (Tr1) cell responses to co-administered antigens, with the polarisation of antigen-specific antibodies to the IgG1 subclass. Studies with innate immune cells *in vitro* indicated that these adaptive immune responses were mediated via the modulation of DC and macrophage activation. Treatment of LPS-stimulated macrophages or murine bone marrow derived DC with CyaA suppressed production of the pro-inflammatory cytokines IL-12p70, TNF-α and the chemokine CCL3 (MIP-1α) and enhanced production of IL-10. Furthermore, CyaA enhanced surface expression of the costimulatory molecule CD80 on immature DC, but inhibited of LPS-induced expression of ICAM-1 (CD54) and CD40, indicating that CyaA selectively modulates DC activation and maturation.

The enzymatic domain of CyaA is located in the 400 NH_2-terminal amino acids, while interaction of CyaA with CD11b/CD18 and with eukaryotic membranes is mediated through the 1306 residue COOH-terminal region of the protein, that has

similarity with the RTX family of bacterial pore-forming toxins (Guermonprez *et al.*, 2001, Ladant and Ullmann, 1999). This family of toxins requires amide-linked fatty-acylation by a toxin-specific accessory protein for function, as originally demonstrated for the *Escherichia coli* haemolysin, HlyA (Issartel *et al.*, 1991). Likewise, acylation of CyaA by its specific accessory protein, CyaC, is necessary for the toxin to lyse RBC, J774 macrophages and Jurkat T-cells (Hewlett *et al.*, 1993, Khelef and Guiso, 1995). CyaA purified from *B. pertussis* is exclusively palmitoylated at lysine 983 (Lys^{983}) (Hackett *et al.*, 1994), while recombinant CyaA expressed in *E. coli* is acylated at Lys^{983} and Lys^{860} in a CyaC-dependent manner (Hackett *et al.*, 1995). Differences in the acylation pattern have been found to alter the haemolytic and pore-forming activity of CyaA, but not its capacity to deliver the enzymatic domain into the cell cytosol (Benz *et al.*, 1994, Hackett *et al.*, 1995, Sebo *et al.*, 1991). The channel-forming activity of CyaA exhibits a cooperative concentration-dependence curve, indicative of protein oligomerisation, while intracellular cAMP accumulation can be triggered by protein monomers (Gray *et al.*, 1998, Guermonprez *et al.*, 2001). These differences have led researchers to suggest that cell lysis and intracellular cAMP accumulation occur through separate mechanisms.

The aim of this study was to examine the role of acylation and of the enzymatic activity of CyaA in its lytic, adjuvant and immunomodulatory effects. The findings demonstrated that an enzymatically inactive mutant of CyaA was devoid of immunomodulatory activity, but retained the ability to induce cell lysis and caspase-3 activation. In contrast, a non-acylated toxin, like the wild-type toxin, modulated DC maturation and cytokine production and acted as an adjuvant for promoting Th2 and Tr cell responses to co-injected antigens *in vivo*, but had limited ability to induce cell death and caspase-3 activation in macrophages.

5.2 Results

5.2.1 Intracellular cAMP induction by CyaA is influenced by but not dependent on acylation.

A-CyaA and NA-CyaA were expressed and purified from *E. coli* harbouring the expression plasmids pJR2 and pJR1 respectively and iAC-CyaA was expressed and purified from *E. coli* harbouring pNM2. As described in chapter 3, the three proteins were purified by ion exchange, affinity chromatography and dialysis with very low levels of LPS associated with each of the three proteins. Both A-CyaA and NA-CyaA were enzymatically active, exhibiting statistically similar specific activities of 126.85 ± 29.9 and 113.0 ± 11.31 μmol camp/min/mg respectively in a cell free assay, while the iAC-CyaA was devoid of enzyme activity (Fig. 5.1A). These results are within the spectrum of those reported from other laboratories (Gray *et al.*, 1998, Khelef *et al.*, 2001, Macdonald-Fyall *et al.*, 2004, Masin *et al.*, 2004). There is considerable variability in the activities of recombinant CyaA reported in the literature, with activities of 117, 194, 330, 400, 408 and 1770 μmol cAMP / mg / min reported from different laboratories (Gentile *et al.*, 1990, Gray *et al.*, 1998, Khelef *et al.*, 2001, Macdonald-Fyall *et al.*, 2004, Masin *et al.*, 2004). However these were based on a variety of different assay systems and some cases different reaction temperatures, which have been shown to affect activity (Gray *et al.*, 1998, Macdonald-Fyall *et al.*, 2004). Using the methods here an activity of 150 μmol cAMP / mg / min for a commercially available preparation of CyaA (List Laboratories Ltd) was recorded while the suppliers had reported an activity of 600 μmol cAMP / mg / min for this batch using a different assay. Therefore, while the activity of the CyaA preparations used in these experiments may be at the lower end of the spectrum of those reported in various laboratories, this must be interpreted with caution, as activity is influenced by the assays employed and the enzyme immunoassay used here may if anything under-estimate activity.

J774 macrophages and DC, both of which express CD11b/CD18, were used to test the role of acylation and enzymatic activity in CyaA-induced intracellular cAMP accumulation. The enzymatically active A-CyaA and NA-CyaA increased the intracellular concentration of cAMP in J774 cells 1000-3000–fold, but iAC-CyaA did not alter cAMP concentration (Fig. 5.1A). A similar pattern of intracellular cAMP induction by the different toxins was observed in DC; 2593 ± 165, 972 ± 350 and 0 pmol cAMP/10^6 cells for A-CyaA, NA-CyaA and iAC-CyaA respectively. While NA-

CyaA increased cAMP concentrations in macrophages over a 10 fold concentration range (Fig. 5.1B), the increases in cAMP concentrations induced by A-CyaA were greater over the same concentration range. Nevertheless, these results clearly demonstrate that acylation is not essential for induction of intracellular cAMP, but it does augment the cAMP concentration generated by CyaA.

5.2.2 Acylation, adenylate cyclase activity and binding to CD11b are required for CyaA induced lysis and caspase-3 activation in macrophages

The ability of these proteins to cause lysis of macrophages was assayed by measuring the release of LDH from cells and viability of treated cells using the AQious Assay. Using the LDH release assay it was determined that A-CyaA and to a lesser extent iAC-CyaA, induced cell death in J774 macrophages, however only minimal lysis was detected with NA-CyaA (Fig. 5.2A). Addition of a blocking anti-CD11b antibody, but not a control antibody to the culture significantly reduced the cell death induced by CyaA (Fig 5.2B). Following incubation with polymyxin B, the low level of lysis detected with 10 μg/ml of NA-CyaA was abrogated and the lysis induced with A-CyaA was reduced (Fig. 5.2C). LPS alone at 200 pg/ml, which is the maximum present in the toxin preparations, caused low but significant cell death. Thus, acylation and binding to CD11b, but not enzyme activity, is required for CyaA to lyse cells, moreover, LPS can augment this lytic effect.

The difference in cytotoxicity between A-CyaA and NA-CyaA was confirmed by examination of the morphology of CyaA-treated macrophages. The morphology of cells treated with three different concentrations of NA-CyaA was similar to that of untreated cells (Fig. 5.3). In contrast, J774 cells treated with A-CyaA at concentrations of 3 or 5 μg/ml (Fig. 5.3 B and C) take on apoptotic morphology, with cytoplasmic and nuclear shrinkage as well as cellular fragmentation. This compares with the obvious apoptotic morphology induced by cycloheximide (Fig. 5.3E). At higher concentrations of A-CyaA (10 μg/ml; Fig. 5.3D) a phenotype more characteristic of necrosis is apparent, with cytoplasmic and nuclear swelling visible and obvious vacuolisation of the cytoplasm.

It has previously been reported that CyaA induced apoptosis in macrophages (Khelef and Guiso, 1995, Khelef *et al.*, 1993). A key step in apoptosis is the activation of the protease, caspase-3. A-CyaA and iAC-CyaA, but not NA-CyaA induced caspase 3 activation (Fig. 5.4A). A-CyaA at concentrations of 5 and 10 μg/ml induced high levels of caspase-3 activity and 3 μg/ml induced lower levels of activation (Fig. 5.4B). In contrast, the caspase 3 activation curves for NA-CyaA are almost indistinguishable over all concentrations tested. These results indicate that acylation, but not enzymatic activity is required for CyaA-induced apoptosis.

5.2.3 Enzymatic activity, but not acylation, is required for modulation of macrophage and DC cytokine production.

The influence of acylation and enzymatic activity on the ability of CyaA to modulate macrophage and DC activation was examined. J774 macrophages or immature bone marrow-derived DC were treated with either CyaA alone (1 μg/ml), CyaA together with LPS (10 ng/ml) or CyaA and polymyxin B (10 μg/ml); addition of polymyxin B was designed to negate any effects of the low concentrations of remaining LPS in the protein preparations. The concentrations of secreted IL-10 and TNF-α were quantified in supernatants 4h later. A-CyaA and NA-CyaA proteins induced IL-10 production by DC (Fig 5.5A) and macrophages (Fig. 5.5B). Addition of polymyxin B, which chelates LPS, abrogated IL-10 production by DC and significantly reduced IL-10 production by macrophages. 200 pg/ml LPS alone, which approximates to the concentrations present in the CyaA protein preparations used for this experiment (191 and 184 pg/ml for A-CyaA and NA-CyaA respectively), failed to induce IL-10 production, indicating that A-CyaA or NA-CyaA do not induce IL-10 production alone but can synergise with LPS for the induction of this cytokine from innate cells. This synergy was evident with LPS at the concentrations present in the protein preparation and was augmented in DC by addition of exogenous LPS (10 ng/ml), but was not significantly altered in macrophages. NA-CyaA and A-CyaA inhibited LPS-induced TNF-α secretion by macrophages and DC. In contrast, iAC-CyaA did not significantly enhance IL-10

production or inhibit TNF-α production (Fig. 5.5), indicating that adenylate cyclase activity is essential for CyaA to modulate cytokine production by macrophages. Unlike the enzymatically active A-CyaA and NA-CyaA, when macrophages or DC are treated with iAC-CyaA in the absence of polymysin B it fails to synergise with the low levels of LPS in the preparation of iAC-CyaA to induce IL-10 from these calls (Fig. 5.5). Conversely, iAC-CyaA is unable to inhibit the TNF-α production from DC and macrophages induced by this low level of LPS co-purified with the toxins when the cells are treated with iAC-CyaA in the absence of polymyxin B, whereas when cells are treated with the enzymatically active toxins, their ability to induce cAMP allows them suppress TNF-α production induced by the co-purified LPS, even in the absence of polymyxin B.

In order to further investigate the role of acylation in the immunomodulatory activity of CyaA, we examined the immunomodulatory effect of the proteins over a range of concentrations (0.1 to 3 μg/ml) (Fig. 5.6). At concentrations of 1 μg/ml and above NA-CyaA and A-CyaA exert similar effects, synergising with LPS to induce IL-10 production and inhibiting LPS-induced TNF-α production by macrophages, while at lower concentrations NA-CyaA had significantly less modulatory activity than A-CyaA. These data indicate that acylation is not an absolute requirement for CyaA to modulate macrophage cytokine release, but it does increase its efficiency.

5.2.4 Non-acylated-CyaA modulates the effects of CpG-ODN signalling through TLR-9

In order to determine whether the synergistic and inhibitory effects of CyaA on IL-10 and proinflammatory cytokine production are limited to LPS, which is closely associated with the protein, or extend to other TLR ligands, DC were treated with the TLR-9 ligand CpG-ODN in the presence of CyaA and the production of cytokines were examined by ELISA. To eliminate any possible influence of LPS, these studies were performed with DC generated from TLR-4-defective C3H/HeJ mice. A-CyaA, NA-CyaA and iAC-CyaA did not stimulate production of IL-10, TNF-α, IL-12p70 or

CCL3 by C3H/HeJ DC. In contrast, CpG-ODN-induced TNF-α, IL-12p70 and CCL3 production by C3H/HeJ DC and this was suppressed by A-CyaA and NA-CyaA, but not by iAC-CyaA (Fig. 5.7). IL-10 production could not be detected 2 h after stimulation of DC with CpG-ODN alone, however addition of A-CyaA or NA-CyaA, but not iAC-CyaA, stimulated significant IL-10 production. These findings demonstrate that the immunomodulatory effects of CyaA are not confined to, or dependent on, signalling of LPS through TLR-4, but can be mediated by the binding of other TLR ligands, such as CpG-ODN, to their receptors. Furthermore, these effects are dependent on enzymatic activity, but are not dependent on acylation of CyaA.

5.2.5 CyaA induced immunomodulatory effects on innate cells are mediated through CD11b

To determine whether the cytokine modulatory effects of A-CyaA and NA-CyaA are mediated by their binding to cells via CD11b/CD18, J774 macrophages were treated with the CyaA proteins and CpG-ODN in the presence of the anti-CD11b antibody, M1/70, which has previously been shown to block the binding of A-CyaA to host cells (Guermonprez *et al.*, 2001). Addition of the anti-CD11b antibody reversed the ability of A-CyaA and NA-CyaA to suppress CpG-ODN-induced production of TNF-α and significantly reduced their ability to synergise with CpG-ODN to enhance IL-10 secretion (Fig. 5.8). Blockling the interaction of A-CyaA and NA-CyaA with CD11b also reduced the ability of these proteins to modulate TLR-4 stimulated cytokine production from macrophages. Incubation of macrophages with the anti-CD11b antibody significantly reduced the amount of IL-10 produced when cells were stimulated with A-CyaA or NA-CyaA in the presence of LPS (Fig. 5.9). Interestingly the amount of TNF-α produced by macrophages after stimulation for 4 hours with LPS (Fig 5.9) was much less than the level detected after stimulation with CpG-ODN (Fig. 5.8). However, A-CyaA reduced this LPS-induced TNF-α production to near zero. However NA-CyaA, which lacks acylation, did not significantly alter the LPS-induced TNF-α production from macrophages at this time point. Interestingly, co-incubation of A-CyaA with LPS in the presence of anti-CD11b antibody not only blocked the

suppression of LPS-induced TNF-α by A-CyaA but infact resulted in a significant increase in TNF-α production over that produced by LPS alone or co-incubation with LPS and A-CyaA. Similarly, co-incubation of macrophages with NA-CyaA and LPS in the presence of the anti-CD11b antibody resulted in a significant increase in TNF-α production by these cells. This suggests a synergistic effect upon TNF-α production by blocking CD11b with this antibody, ligation of TLR-4 by LPS and stimulation with CyaA. Indeed, LPS-induced TNF-α from macrophages is significantly increased when cells are also treated with the anti-CD11b antibody (Fig 4.9) These data demonstrate that modulation of TLR-ligand induced cytokine release from macrophages by A-CyaA and NA-CyaA involves interaction with the CD11b/CD18 intigrin, and specific uptake of both these proteins into innate immune cells through this cell surface receptor.

5.2.6 Influence of acylation and enzymatic activity on the modulation of DC maturation by CyaA

Maturation of DC in response to pathogen-derived molecules, such as LPS and CpG-ODN, can be detected by changes in cell surface expression of co-stimulatory molecules. Here the effect of acylation and enzymatic activity of CyaA on its ability to induce maturation of immature bone-marrow-derived DC or to modulate CpG-ODN-induced maturation of DC was examined by FACS. The effects of A-CyaA and its derivatives were tested in the presence of polymyxin B in order to rule out any effect of the low levels of LPS associated with the preparations of the proteins. Both NA-CyaA and A-CyaA enhanced DC surface expression of CD80 and suppressed endogenous expression of ICAM-1 and CD40. Furthermore, pre-incubation of DC with NA-CyaA and A-CyaA suppressed CpG-ODN-induced expression of CD40 and ICAM-1 (Fig. 5.10). In contrast, iAC-CyaA had little effect on cell surface marker expression on DC, marginally enhancing ICAM-1. A-CyaA and NA-CyaA also modulated LPS-induced expression of CD40 and ICAM-1 on DC from BALB/c mice (Fig. 5.11). These data demonstrate that CyaA modulates DC maturation in response to TLR-4 and TLR-9 ligands and that enzymatic activity, but not acylation, is essential for these effects.

5.2.7 Acylation is not required for the adjuvant activity of CyaA

Having demonstrated that NA-CyaA and A-CyaA exert similar effects on innate cells and that iAC-CyaA lacks immunomodulatory activity *in vitro*, experiments were performed to determine the influence of acylation and enzymatic activity on the adjuvant effect of CyaA in BALB/c mice were immunised once or twice with KLH alone or with A-CyaA, NA-CyaA or iAC-CyaA. Analysis of antibody titres revealed that NA-CyaA and A-CyaA enhance KLH-specific IgG responses to the same extent, whereas iAC-CyaA had significantly reduced adjuvant activity (Fig. 5.12B). A single dose of 1 or 5 μg/ml of A-CyaA or NA-CyaA augmented KLH-specific IgG, whereas 0.2 μg of these preparations or 0.2, 1 or 5 μg iAC-CyaA had little or no adjuvant activity (Fig. 5.12A). The adjuvant effect is unlikely to be due to residual LPS in the toxin preparations as enhancement of antibody response to KLH was also observed in TLR-4-defective C3H-HeJ mice (Fig. 4.7). Although iAC-CyaA did significantly enhance antibody responses to KLH after two immunization, the IgG titres were significantly lower than that observed with the wildtype toxin (Fig. 5.12B). We also assessed antigen-specific cytokine production by popliteal lymph node cells after a single immunization with KLH and CyaA or NA-CyaA. KLH-specific cytokine production was low or undetectable in lymph node cells from mice immunised with KLH alone (Fig. 5.13). However lymph node cells from these mice did proliferate and secrete IL-4, IL-5, IL-10 and IFN-γ in response to PMA and anti-CD3. In contrast, antigen-specific proliferation and IL-4, IL-5 and IL-10 production (Fig. 11) were detected in lymph node cells from mice immunised with KLH in the presence of A-CyaA or NA-CyaA. IFN-γ was produced by lymph node cells from mice immunised with KLH in the presence of A-CyaA or NA-CyaA, however, the concentrations were low when compared with that observed in mice immunised with KLH in the presence of CpG-ODN (Fig 4.**2**). This cytokine profile is consistent with the previous demonstration that CyaA preferentially enhances Th2 and Tr1 responses (chapter 4) and with the observation that IgG1 was the dominant subclass of antibody induced with KLH in the presence of A-CyaA or NA-CyaA (Fig. 5.14). Therefore I conclude that lack of acylation does not compromise the adjuvant properties of CyaA or its ability to direct the induction of T cell and antibody responses *in vivo*.

5.3 Discussion

The results here show that posttranslational modification of CyaA by acylation is required for optimal caspase-3 activation and cell death in macrophages, but is not essential for CyaA to modulate innate immune cell activation *in vitro* or to act as an adjuvant *in vivo*. Non-acylated CyaA, like acylated CyaA, enhanced macrophage and DC production of the anti-inflammatory cytokine, IL-10 and inhibited the production of the proinflammatory cytokines TNF-α and IL-12 and the chemokine CCL3 in response to TLR ligands. In contrast, a mutant toxin with substitutions in the adenylate cyclase active site rendering it enzymatically inactive failed to modulate DC or macrophage activation, indicating that enzymatic activity and consequent cAMP accumulation were required for the *in vitro* immunomodulatory effects of CyaA. Significantly, and in contrast to previous reports on other cell types (El-Azami-El-Idrissi *et al.*, 2003, Hewlett *et al.*, 1993), these data demonstrate that, while the absence of acylation reduced, it did not prevent CyaA-induced cAMP accumulation in macrophages and DC. These findings demonstrate for the first time that acylation is not essential for the immunomodulatory effects of CyaA and that these effects are principally mediated by adenylate cyclase-induced intracellular cAMP.

CyaA is a member of the RTX family of pore-forming proteins, but it has a unique bifunctional nature, with an enzymatic domain located in the NH_2-terminal and the RTX domain in the COOH-terminal. This domain arrangement allowed us to dissect the separate activities of CyaA - cell lysis, cAMP accumulation, immunomodulation, apoptosis - in a way that was not possible with any other RTX toxin. Lysis of eukaryotic cells by RTX toxins is dependent on post-translational acylation, as initially demonstrated for the *E. coli* haemolysin, HlyA (Issartel *et al.*, 1991, Narayanan *et al.*, 2002). Likewise acylation of the RTX domain of CyaA is considered to be necessary for its haemolytic and cytotoxic activity (El-Azami-El-Idrissi *et al.*, 2003, Hewlett *et al.*, 1993, Khelef and Guiso, 1995). Previous studies have suggested that acylation is required for efficient adjuvanticity of CyaA, while the increase in intracellular cAMP levels generated by the adenylate cyclase domain is responsible for its immunomodulatory effects (Bagley *et al.*, 2002b, Khelef and Guiso, 1995, Khelef *et al.*, 1993). These data demonstrate that acylation is not essential for

CyaA to induce cAMP accumulation in macrophages or to modulate activation of macrophages and DC. Like A-CyaA, NA-CyaA synergised with LPS to enhance secretion of IL-10, while inhibiting TNF-α and IL-12 p70 production. LPS alone at the concentration present in the toxin preparations did not induce IL-10 production. Likewise the toxins alone did not induce IL-10 in DC from TLR-4 defective mice or in macrophages or DC from normal mice in the presence of polymyxin B. However when combined, the toxin and TLR ligand synergised to promote IL-10 production. The modulatory effects of A-CyaA and NA-CyaA on innate cell cytokine production were not confined to the TLR-4 ligand, LPS, which forms a complex with CyaA (Munier *et al.*, 1992), but were also observed with the TLR-9 ligand, CpG-ODN. This suggests that a physical association between the two modulators (as in the case of CyaA and LPS) is not essential for the synergy, but possibly binding to two distinct receptors is involved. This hypothesis is supported by previous studies showing synergy between TLR and other signalling pathways (Ladant *et al.*, 1986, Lavelle *et al.*, 2003). NA-CyaA and A-CyaA also had similar effects on DC maturation, upregulating CD80 expression, while downregulating CD40 and ICAM-1 expression. Furthermore NA-CyaA, like A-CyaA, was an effective adjuvant *in vivo* when co-injected with a foreign antigen, stimulating the production of antigen-specific IgG1 antibodies and IL-4, IL-5 and IL-10 production by T-cells.

Unlike acylation, enzyme activity was required for maximal adjuvant and immunomodulatory activity of CyaA. A CyaA mutant lacking functional adenylate cyclase activity was unable to cause an increase of intracellular cAMP concentrations, did not modulate DC maturation and was unable to suppress LPS-induced TNF-α release or to enhance IL-10 production by innate immune cells. This suggests that increased intracellular cAMP concentrations are involved in modulation of innate immune cell activation by CyaA. This conclusion is consistent with the observations that increases in intracellular cAMP concentration by stimuli such as cholera toxin or forskolin (Bagley *et al.*, 2002b, Lavelle *et al.*, 2003), lead to suppression of proinflammatory cytokines and chemokines, such as IL-12 and CXCL10 and enhancement of IL-10 and CCL17.

RTX toxins bind to leucocytes through β_2 integrins and CD11b/CD18 has been identified as the receptor for CyaA (El-Azami-El-Idrissi *et al.*, 2003, Guermonprez *et al.*, 2001). In order to bind RBC, which lack β_2 integrins, CyaA and other RTX proteins require acylation (Hewlett *et al.*, 1993). However the role of acylation in the binding of RTX toxins to purified β_2 integrins has not been reported. The results demonstrate that acylation is not essential for CyaA to induce cAMP accumulation in innate immune cells expressing CD11b/CD18. Although cAMP induction was reduced with the non-acylated toxin, which may be due to reduced stability of interaction with CD11b (Guermonprez *et al.*, 2001) or less effective penetration of the cell membrane compared with acylated native toxin (Hewlett *et al.*, 1993, Issartel *et al.*, 1991), it did induce significantly increased levels of intracellular cAMP in macrophages and DC. Previous studies had suggested that acylation of CyaA was essential for the induction of increased cAMP levels in eukaryotic cells. However, these studies had not employed innate immune cells but rather RBC and Jurkat T cells, each of which express low or no CD11b/CD18, and CHO cells transfected with CD11b (El-Azami-El-Idrissi *et al.*, 2003, Hewlett *et al.*, 1993). Previous studies have demonstrated that acylation is important for binding of CyaA to RBC, but that interaction with CD11b-expressing cells is via residues 1166 to 1281 (El-Azami-El-Idrissi *et al.*, 2003). CyaA proteins containing mutations within this region bind red blood cells, but not CD11b-expressing cells. Conversely, a CyaA protein with a FLAG epitope insert at amino acid 926 bound CD11b-expressing cells, but not red blood cells. The authors suggested that this was due to the disruption of the structure required for acylation of the adjoining Lys^{983}. In addition, CyaA binding to CD11b expressing cells is saturable, while binding to RBC is not (Guermonprez *et al.*, 2001, Martin *et al.*, 2004). These data suggest that CyaA may interact with RBC and CD11b-expressing cells by two different mechanisms. It is likely that the acyl groups also associate with the eukaryotic cell membrane increasing the efficiency of CyaA-host cell interactions and facilitating the transmembrane delivery of the adenylate cyclase domain into target cells. This interaction may be of increased importance for cells lacking CD11b/CD18 and may explain the failure of NA-CyaA to deliver the adenylate cyclase domain into red blood cells, Jurkat T cells and CD11b transfected CHO cells. In the present study we have demonstrated that

acylation is not essential for CyaA-induced cAMP accumulation and immunomodulation in macrophages and that anti-CD11b antibodies block the modulatory effects of A-CyaA and NA-CyaA. Together these data indicate that posttranslational modification is not essential for interaction of CyaA with its β_2 integrin receptor CD11b/CD18 on the host cell surface. Interaction of CyaA with CD11b/CD18, which is primarily expressed on macrophage and myeloid DC, may be a strategy evolved by the bacteria to target and disrupt cytokine signalling pathways in key cells of the innate immune system.

CyaA oligomerises in the cell membrane to form pores and thus cause membrane disintegration. The data here demonstrate that in cells expressing the CyaA receptor CD11b/CD18, such as DC and macrophages, acylation is important for cytotoxicity. CyaA induces cell death in macrophages, as indicated by the DNA fragmentation seen in CyaA-treated cells *in vitro* (Khelef and Guiso, 1995). CyaA-deficient *B. pertussis* does not induce lysis of J774 macrophages, has reduced capacity to cause apoptosis of alveolar macrophages *in vivo* and is considerably less virulent in mice (Goodwin and Weiss, 1990, Khelef *et al.*, 1993). In these experiments cell death was observed in macrophages incubated with A-CyaA or iAC-CyaA. In contrast cell lysis was only observed with the highest dose of NA-CyaA examined and this effect was abolished, as was a proportion of the lytic activity of A-CyaA, by co-incubation with polymyxin B. LPS and LPS-induced TNF-α have been associated with apoptosis (Hull *et al.*, 2002) and may therefore have contributed to the cell death observed with NA-CyaA and CyaA. This may explain the higher levels of cell lysis reported in previous studies, where steps were not taken to remove LPS during the purification of CyaA. These experiments also provide evidence that CyaA induces activation of caspase-3, a key effector molecule in apoptosis. Caspase-3 activation was observed in macrophages treated with the wild-type toxin and with the enzymatically inactive mutant, but not with NA-CyaA. Overall our findings suggest that acylation, but not adenylate cyclase activity, facilitates CyaA-induced cell death.

This study provides new insights into the role of acylation of RTX domains in the immunomodulatory and pro-apoptotic activities of bacterial toxins and has addressed the contribution of LPS, present at high concentrations in many recombinant

CyaA preparations, to its immunological activity. The non-acylated toxin retains the ability to enhance cAMP, albeit at a slightly lower efficiency than the wildtype toxin. Elevation of intracellular cAMP appears to be a critical factor in the immunomodulatory activity of CyaA, but is not essential for its ability to cause cell lysis or caspase-3 activation. Like the wild-type toxin, this non-acylated derivative specifically targets CD11b/CD18 expressing macrophages and DC, inhibiting the release of pro-inflammatory cytokines and enhancing IL-10 production and activating DC into a semi-mature phenotype that directs the induction of Th2 and IL-10-producing Tr1 cells. Thus, NA-CyaA is capable of promoting both innate and adaptive IL-10 production, without causing cell death, and as a consequence has considerable potential as an adjuvant and immunotherapeutic agent for the prevention and/or treatment of inflammatory and Th1-mediated autoimmune diseases.

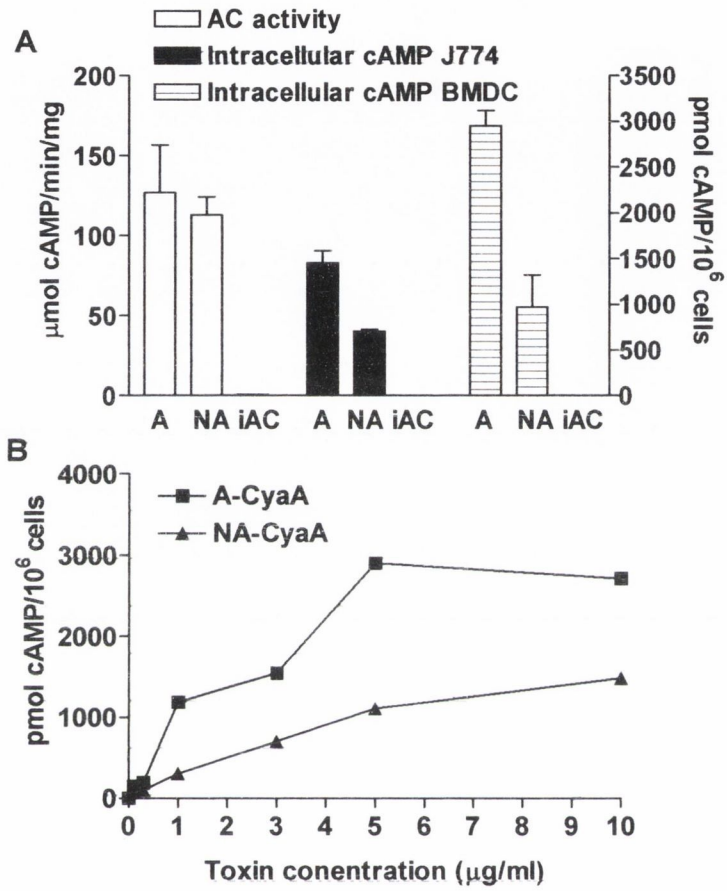

Figure 5.1. Intracellular cAMP induction by CyaA is influenced by, but not dependent on acylation. (A) Cell-free adenylate cyclase activity and intracellular cAMP accumulation in J774 macrophages and BMDC over 60 minutes in response to 1 µg/ml of A-CyaA (A) NA-CyaA (NA) or iAC-CyaA (iAC). (B) Intracellular cAMP accumulation in J774 macrophages over 60 minutes in response to increasing concentrations of A-CyaA and NA-CyaA

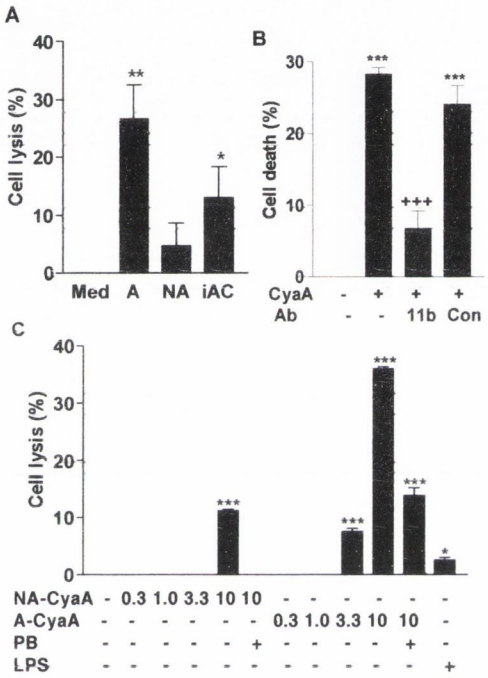

Figure 5.2. Acylation, adenlyate cyclase activity and binding to CD11b are required for the induction of maximal cell death by CyaA. (A) Lysis of J774 cells, determined by LDH release assay, in response to A-CyaA, NA-CyaA or iAC-CyaA (10 μg/ml) or medium only (Med) (B) Cell death of J774 cells, determined by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay in response to A-CyaA (10 μg/ml), in the absence or presence of anti-CD11b (11b) antibody or a control antibody (Con). (C) Cell lysis of J774 cells, determined by LDH release assay, in response to increasing doses of A-CyaA and NA-CyaA (0.3-10 μg/ml) in the absence or presence of polymyxin B (PB) or to 200 pg/ml LPS. Results are representative of experiments repeated at least twice. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, versus medium control. +++ $P<0.001$: A-CyaA versus A-CyaA and anti-CD11b.

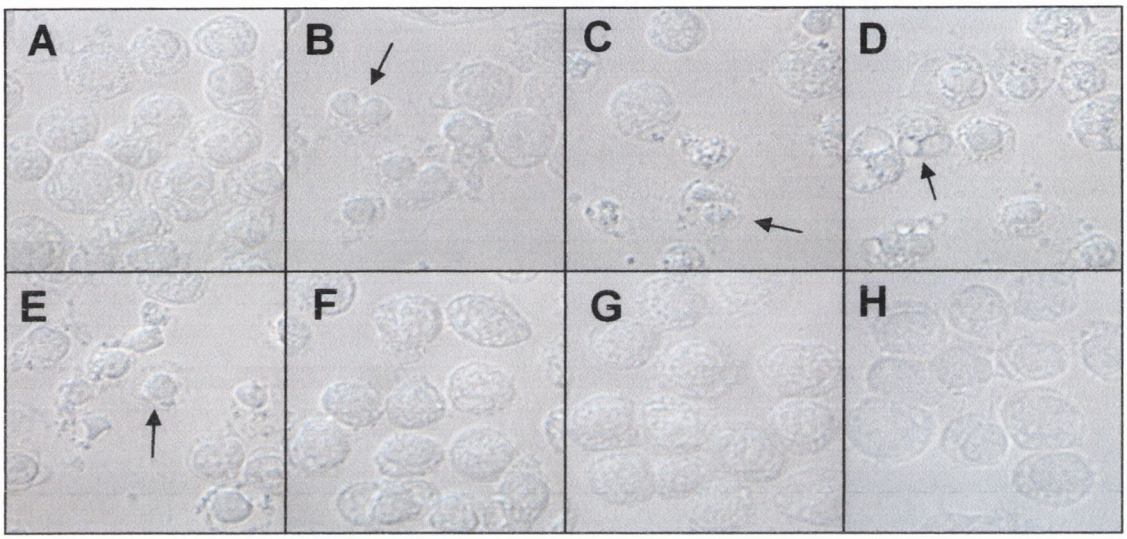

Figure 5.3: Acylation is necessary for CyaA-induced morphological changes in macrophages. J774 macrophages were untreated (A), or treated with 3 (B), 5 (C) or 10 (D) μg/ml A-CyaA, 1 μM cycloheximide (E) or 3 (F), 5 (G) or 10 (H) μg/ml NA-CyaA for 6 h. Altered morphology after treatment with A-CyaA and cycloheximide compared with normal morphology in untreated and NA-CyaA treated cells. Arrows indicate examples of apoptotic/necrotic cells.

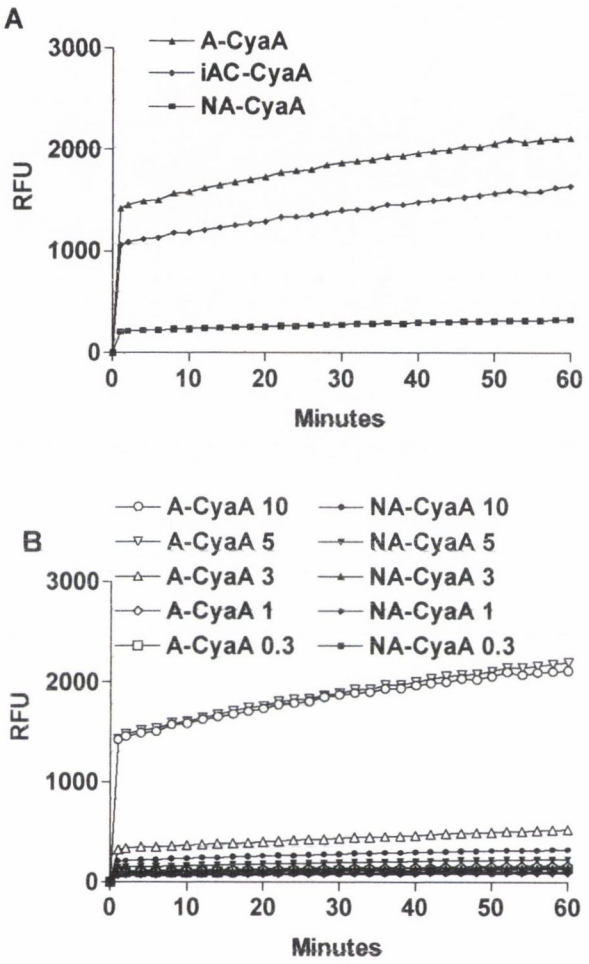

Figure 5.4. CyaA induces caspase-3 activation, which is dependent on acylation. (A) Caspase-3 activation induced by A-CyaA, NA-CyaA or iAC-CyaA (10 μg/ml) in J774 macrophages, expressed as relative fluorescence units (RFU) over time. (B) Caspase-3 activation induced by A-CyaA or NA-CyaA (0.3-10 μg/ml) in J774 macrophages. Results are representative of 3 experiments.

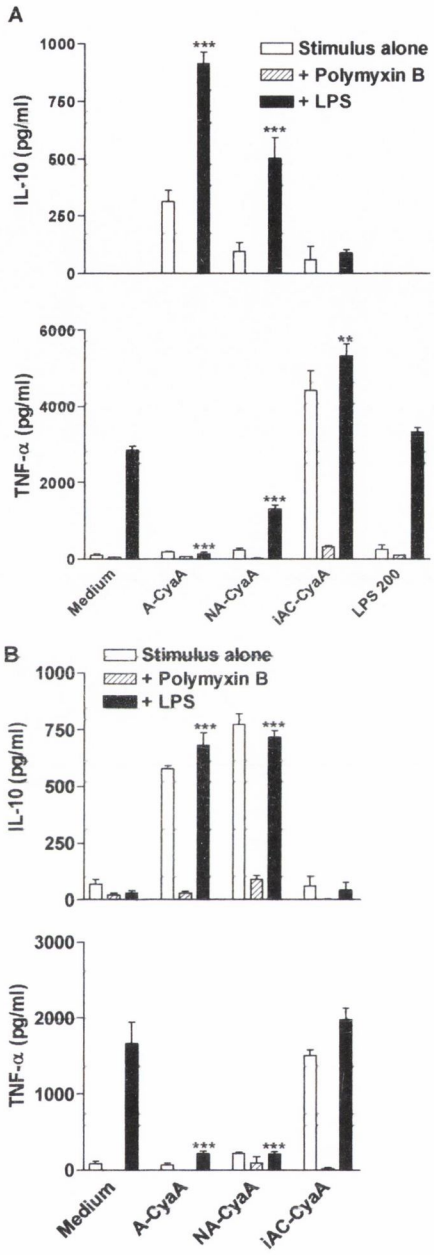

Figure 5.5. Enzymatic activity, but not acylation, of CyaA is required for modulation of macrophage and DC cytokine production. Bone marrow derived immature DC from BALB/c mice (A) or J774 macrophages (B) were incubated with medium only, A-CyaA, NA-CyaA or iAC-CyaA (1 μg/ml) in the presence or absence of 10 μg/ml polymyxin B. 2 h later 10 ng/ml LPS was added as indicated on the figure. DC were also incubated with 200 pg/ml of LPS. After 4 h incubation, supernatants were collected and IL-10 and TNF-α concentrations determined by ELISA. ** $P<0.01$, *** $P<0.001$: CyaA and LPS versus LPS. Results are means ± SD of triplicate assays and are representative of 3 experiments.

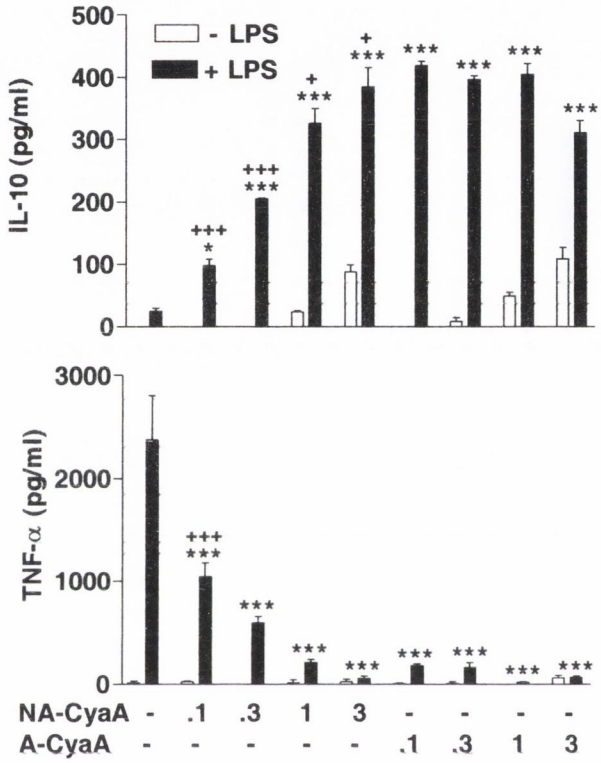

Figure 5.6. Effect of A-CyaA and NA-CyaA toxin concentration on the modulation of macrophage cytokine production. J774 macrophages were treated with A-CyaA and NA-CyaA at the indicated concentrations (μg/ml), with and without the addition of 10 ng/ml LPS 2 hours later. After a further 4 h incubation, supernatants were collected and IL-10 and TNF-α concentrations determined by ELISA. * $P<0.05$, *** $P<0.001$: CyaA and LPS versus LPS. + $P<0.05$, +++ $P<0.001$: A-CyaA versus NA-CyaA at same concentration. Results are means ± SD of a representative experiment performed twice in triplicate.

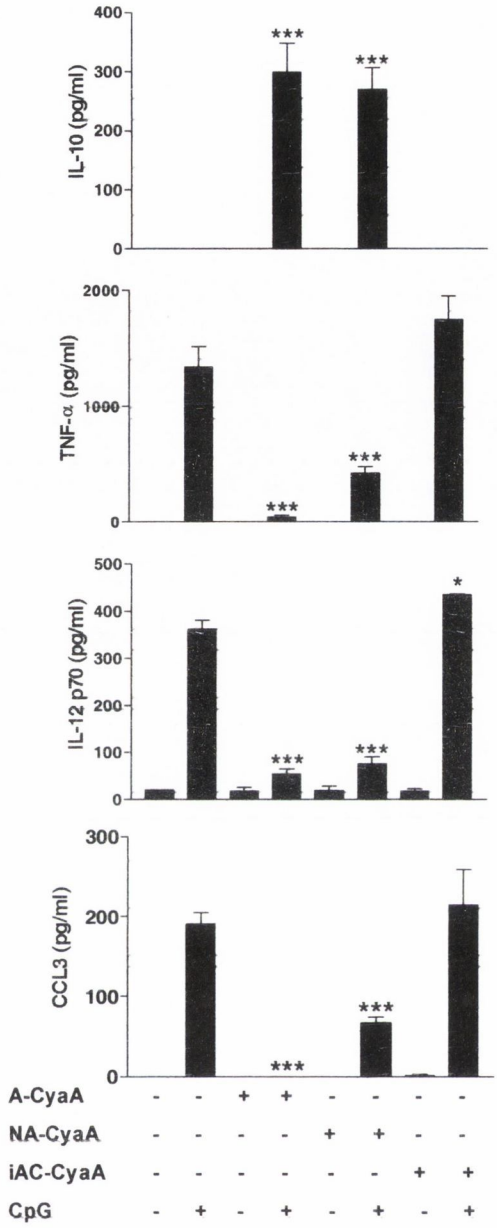

Figure 5.7. Non-acylated CyaA modulates CpG-ODN-stimulated cytokine and chemokine release from DC. DC from C3H/HeJ mice were incubated with 1 μg/ml A-CyaA, NA-CyaA or iAC-CyaA for 2h, before the addition of 10 μg/ml CpG-ODN. Supernatants were tested for the presence of IL-10, TNF-α and CCL3 4h later and for IL-12 p70 24 h later. * $P<0.05$, *** $P<0.001$: CpG-ODN versus CyaA and CpG-ODN. Results are means ± SD of a representative experiment performed twice in triplicate.

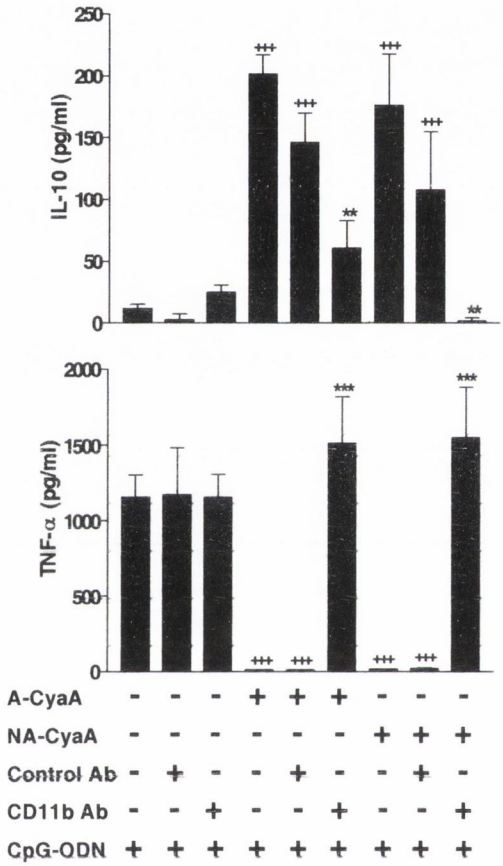

Figure 5.8. CyaA-induced modulation of CpG-ODN macrophage activation is dependent on interaction with CD11b. J774 macrophages were incubated with A-CyaA or NA-CyaA (1ug/ml) in the presence of polymyxin B (10 μg/ml) for 2h before the addition of 10 μg/ml CpG-ODN. Cells were incubated with 10 μg/ml anti-CD11b or isotype control antibody where indicated prior to the addition of CyaA. After a further 4 h incubation, supernatants were collected and IL-10 and TNF-α concentrations determined by ELISA. Results were compared by the one-way ANOVA with Tukey post-test. $^{**}P<0.01$, *** $P<0.001$: anti-CD11b versus control antibody. +++ $P<0.001$: CpG-ODN + toxin versus CpG alone. Results are means ± SD of a representative experiment performed twice in triplicate.

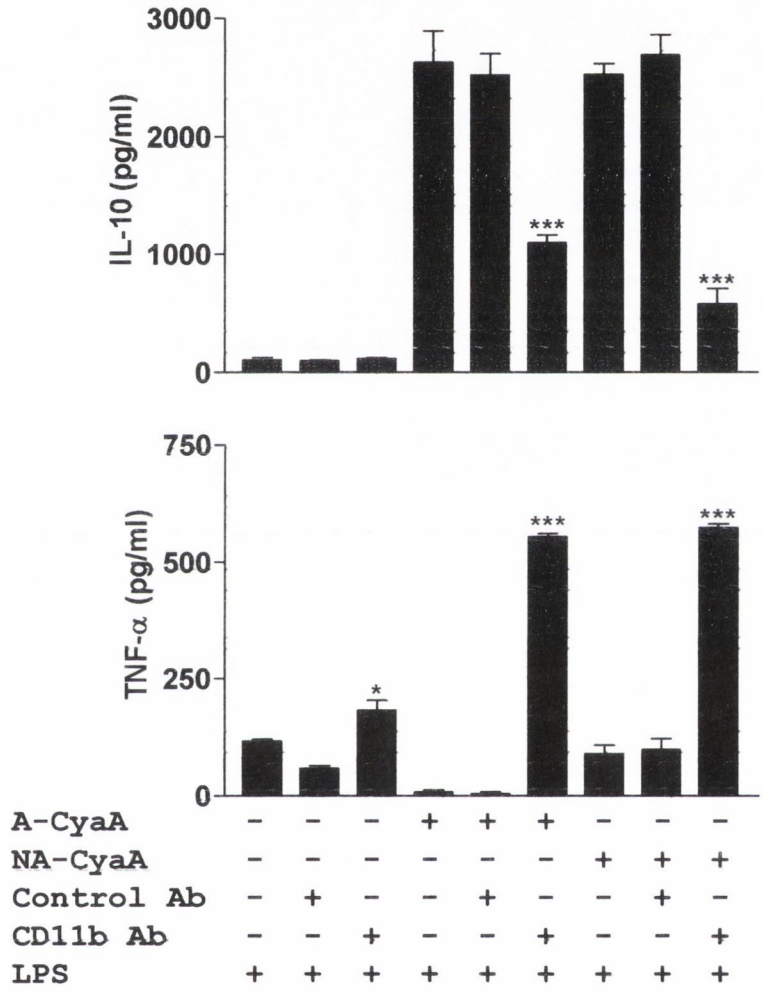

Figure 5.9. CyaA-induced modulation of LPS-induced macrophage activation is dependent on interaction with CD11b. J774 macrophages were incubated with A-CyaA or NA-CyaA (1ug/ml) in the presence or absence of polymyxin B (10 μg/ml) for 2h before the addition of LPS (10 ng/ml). Cells were incubated with 10 μg/ml anti-CD11b or isotype control antibody where indicated prior to the addition of CyaA. After a further 4 h incubation, supernatants were collected and IL-10 and TNF-α concentrations determined by ELISA. Results were compared by the one-way ANOVA with Tukey post-test. *P<0.05, **P<0.01, *** P<0.001: anti-CD11b versus control antibody. Results are means ± SD of a representative experiment performed twice in triplicate.

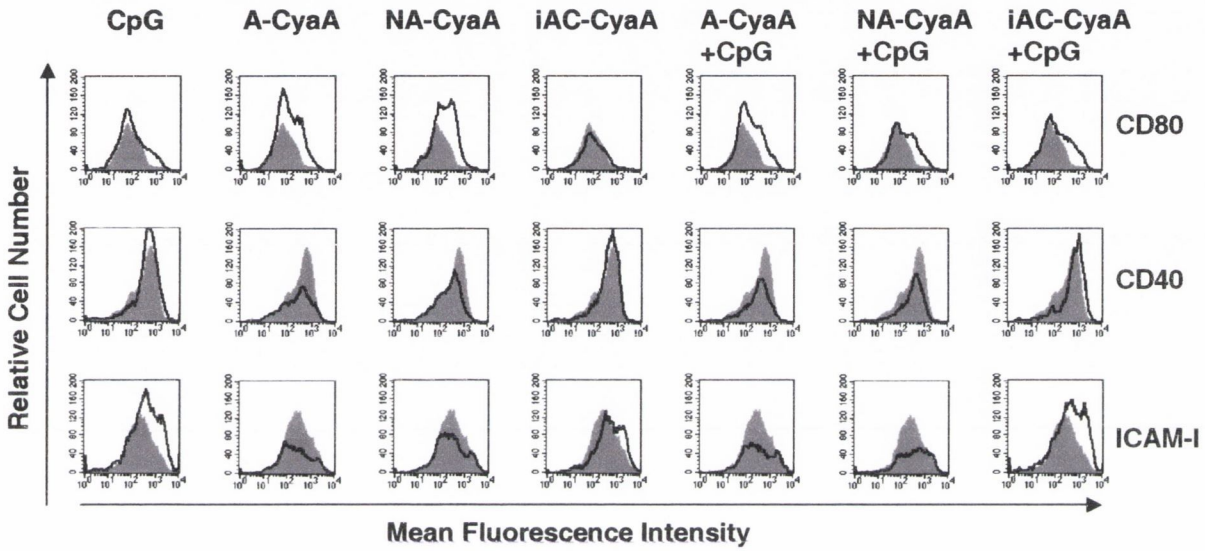

Figure 5.10. Enzyme activity, but not acylation, is required for modulation of CpG-ODN induced DC maturation by CyaA. DC from BALB/c mice were cultured for 24 h with 1 μg/ml NA-CyaA, A-CyaA or iAC-CyaA alone or in the presence of CpG-ODN (10 μg/ml) or with CpG-ODN or medium only. Polymyxin B (10 μg/ml) was added to all cultures to rule out any contribution of residual LPS. Cells were harvested, washed and labelled with anti-CD11c and anti-CD80, anti-CD40, anti-ICAM-1 or the appropriate isotype control antibody. Results of immunofluoresence analysis are shown for treated (black lines) compared with un-treated (grey histograms) DC and are representative of 6 experiments.

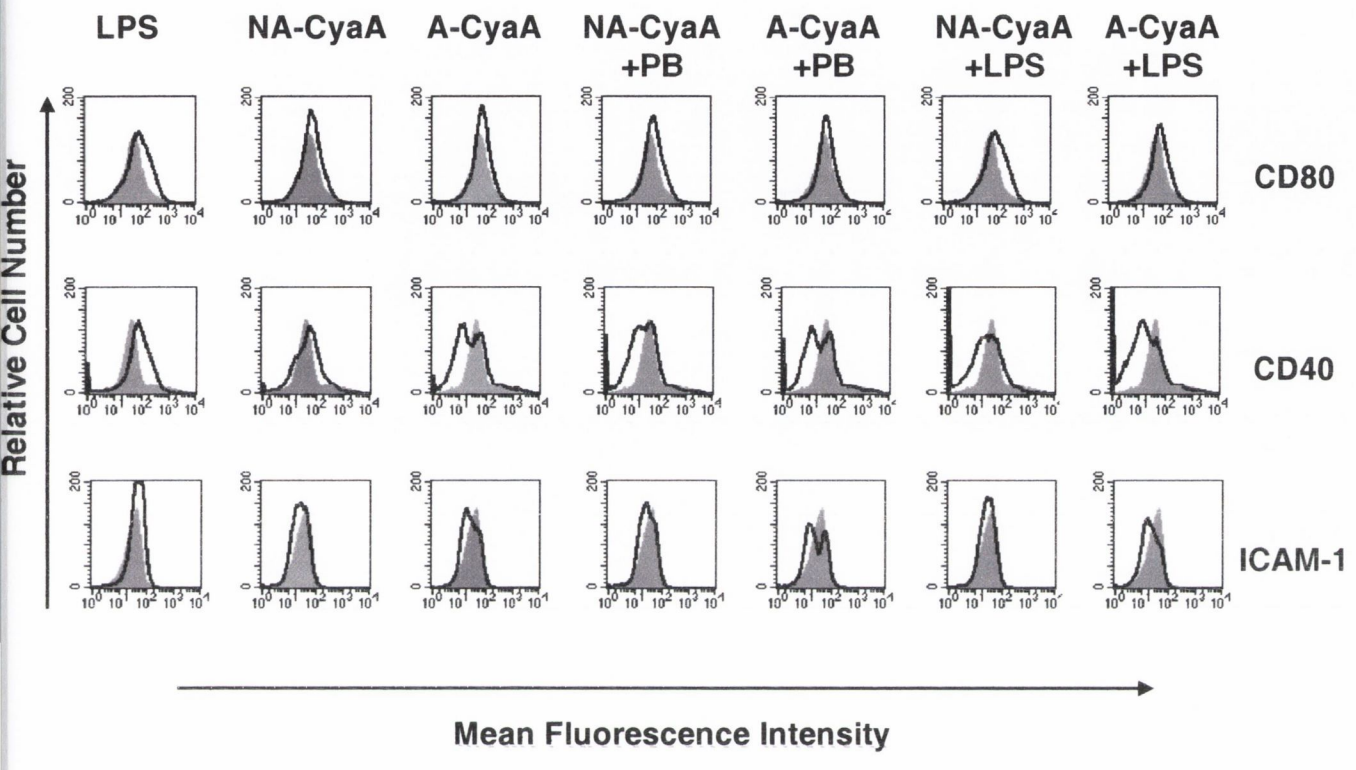

Figure 5.11. Enzyme activity, but not acylation, is required for modulation of LPS induced DC maturation by CyaA. DC from BALB/c mice were treated with 1 μg/ml NA-CyaA or A-CyaA in the presence or absence of 10 μg/ml polymyxin B (PB). 2 h later 1 μg/ml LPS or medium only was added. 24 h later cells were harvested, labelled with biotin-conjugated hamster anti-CD11c IgG and streptavidin-PerCP or isotype control. Cells were simultaneously stained with either FITC-labelled anti-CD40 IgG2a, phycoerythrin-labelled anti-CD80 IgG2k, FITC-labelled anti-ICAM-1 IgG1 or the appropriate isotype control antibody. Results of immunofluoresence analysis are shown for treated (black lines) compared with untreated (grey histograms) DC.

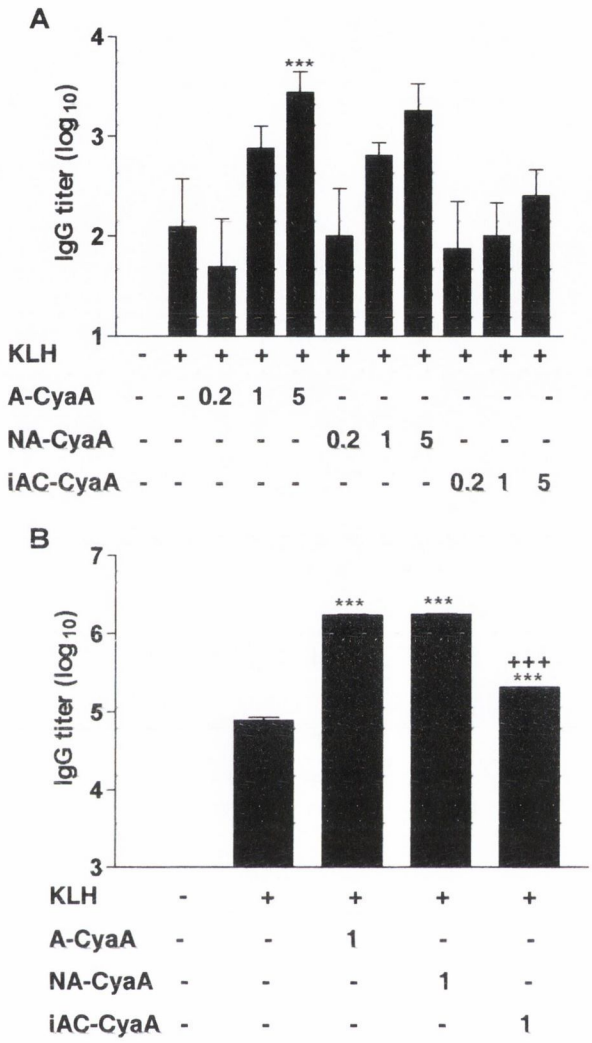

Figure 5.12. Enzymatic activity, but not acylation, influences the adjuvant effect of CyaA. BALB/c mice were immunised once (A) or twice (B) at 0 and 21 days in the footpad with PBS, KLH (5 μg) alone or with NA-CyaA, A-CyaA or iAC-CyaA (0.2-5 μg). Serum was prepared 7 days later and tested for KLH-specific IgG by ELISA (expressed as end point titres). Results are means ± SD for 5 mice, with samples tested in triplicate. *** $P<0.001$, versus KLH alone; +++ $P<0.001$, versus KLH + A-CyaA. Results are representative of 4 experiments, 2 after a single and 2 after a booster dose.

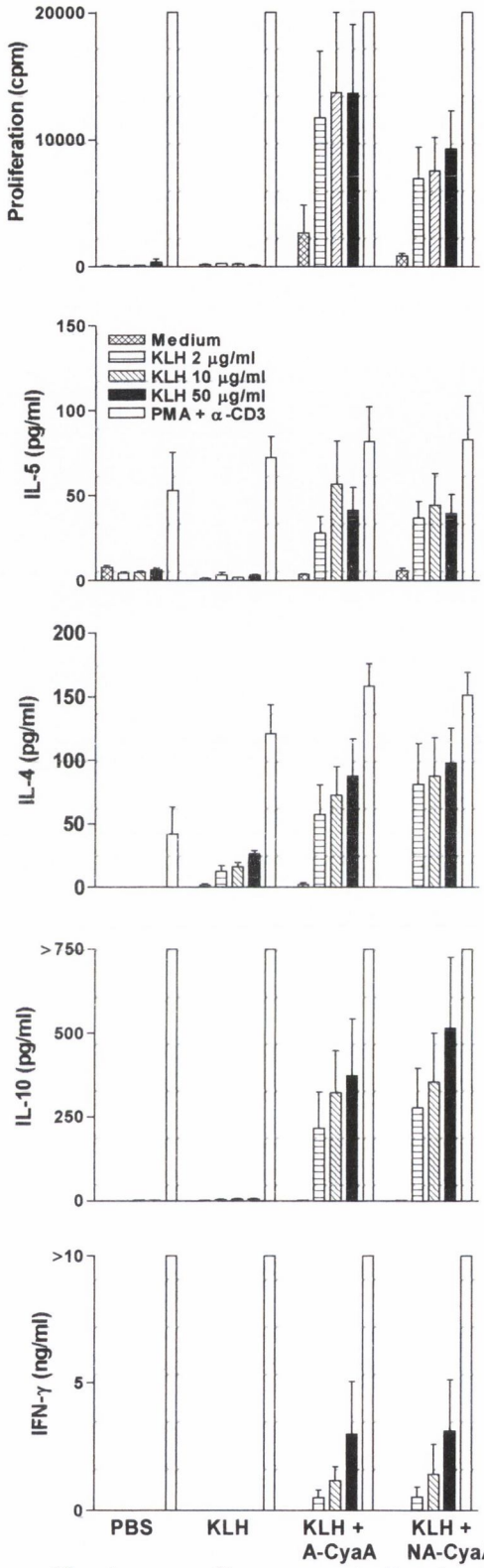

Figure 5.13. NA-CyaA is as effective an adjuvant as A-CyaA, promoting T cell proliferation and enhancing Th2/Tr1 type response to co-administered antigens *in vivo*. Mice were immunised in the footpad with PBS, KLH (5 µg) alone or with NA-CyaA or A-CyaA (1 µg). Popliteal lymph node suspensions were prepared after 7 days and KLH-specific cytokine release determined after 3 days in sulture with antigen (KLH 0-50 µg) and proliferation measured on the forth day. Results are means ± SD for 5 mice, with samples tested in triplicate, and are representative of three experiments.

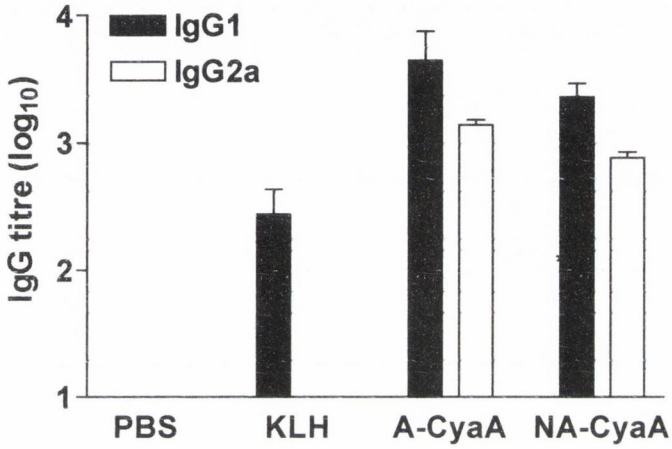

Figure 5.14. Polarised KLH-specific IgG1 imduction by CyaA is not dependant on aclyation. BALB/c mice were immunised in the hind footpad with PBS, KLH (5 μg) alone or with NA-CyaA, A-CyaA (1 μg). Serum was prepared 7 days later and tested for KLH-specific IgG1 and IgG2a by ELISA (expressed as end point titres). Results are means ± SD for 5 mice, with samples tested in triplicate. Results are representative of 2 experiments.

CHAPTER 6

CyaA MODULATES IMMUNE RESPONSES AGAINST CO-ADMINISTERED ANTIGEN WHEN DELIVERED NASALLY

6.1 Introduction

Bordetella pertussis is a Gram-negative bacteria, which is the causative agent of whooping cough. *B. pertussis* causes a chronic and persistent infection of young children and is re-emerging as an important respiratory pathogen of adults in the developed world (Cherry, 2003). *B. pertussis* expresses many virulence factors, including CyaA, which aid its survival and promotes colonisation of the respiratory mucosa and the successful establishment of a persistent infection (Mills, 2001, Smith *et al.*, 2001). CyaA is an A-B type toxin with cell lysis and enzymatic functional domains, and binds the CD11b/CD18 (CR3) integrin via the 1166-1281 amino acid region at the C-terminal of the protein (Guermonprez *et al.*, 2001, Ladant and Ullmann, 1999). By specifically targeting sentinel cells of the immune system, such as DC and macrophages, which express the CR3 integrin CyaA perturbs immune cell functions, including superoxide generation (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987), chemotaxis (Harvill *et al.*, 1999, Khelef *et al.*, 1994) and phagocytosis (Weingart *et al.*, 2000) in addition to causing apoptosis of macrophages (Gueirard *et al.*, 1998, Khelef and Guiso, 1995, Khelef *et al.*, 1993) and cell death in a variety of other cell types including T cells (Lee *et al.*, 1999) and red blood cells (Westrop *et al.*, 1997).

The work presented in chapters 4 and 5 demonstrated that CyaA modulates innate immune cells, promoting the maturation of DC into a phenotype characterised by increased expression of CD80 with low levels of CD40 and ICAM-I, enhanced IL-10 secretion and the suppression of inflammatory cytokine production. This DC phenotype promotes the generation of IL-10 secreting Tr1 cells and Th2 cells. CyaA also promotes IgG1 antibody production against a co-immunised by-stander antigen. CyaA induces caspase-3 activation, a marker of apoptosis, as well as lysing both DC and macrophages. The enzymatic activity of CyaA is necessary for its immunomodulatory activity and binding to CD11b/CD18 is required for both immunomodulation and the induction of cell death in innate cells.

CyaA plays an important role in regulating the immune response during infection by *B. pertussis* at the site of infection. *B. pertussis* lacking CyaA fail to recruit

immune cells into the lungs of infected mice (Harvill *et al.*, 1999, Khelef *et al.*, 1994), do not establish a persistent infection and bacteria are cleared rapidly from the lungs (Goodwin and Weiss, 1990, Gross *et al.*, 1992, Weiss *et al.*, 1984).

Interestingly, *B. pertussis* promotes the TLR-4 dependent induction of regulatory cytokines and cells during infection, (Higgins *et al.*, 2003). In the absence of these Tr1 cells, clearance of *B. pertussis* is delayed and accompanied by severe immunopathology in the lung. Regulatory T cells appear to be induced during chronic or persistent infection either as an evasion strategy by the pathogen to inhibit protective immune responses by the host or as a host protective mechanism to limit damage mediated by immune cells during infection (Mills, 2004). Like FHA (McGuirk *et al.*, 2000), CyaA may promote the induction of Tr1 cells during *B. pertussis* infection. When the immunological activity of CyaA demonstrated in chapter 4 is taken together with the previous reports that CyaA can suppress inflammatory responses from human monocyte-derived DC (Bagley *et al.*, 2002b) and is required for successful *B. pertussis* infection it suggests that CyaA may modulate the immune response at the mucosa and could have potential as a mucosally delivered vaccine adjuvant and immunotherapeutic molecule.

Mucosal vaccines are easier to deliver compared than vaccines delivered by injection, which helps to improve patient compliance, as well as eliminating problems with disposal or re-use of contaminated needles. Mucosal delivery also has the potential to confer protection at the site of infection by inducing local as well as systemic immunity. However, the generation of a sufficiently strong immune response by mucosal vaccination with purified or recombinant protein antigens requires the use of an adjuvant in the vaccine formulation (O'Hagan and Valiante, 2003). While alum is included in many paranterally delivered vaccines, it is not used with mucosal vaccines. Pathogen and plant derived molecules with mucosal adjuvant activity have shown great potential in their ability to stimulate both systemic and local immunity (Giuliani *et al.*, 1998, Lavelle *et al.*, 2001) and to induce antibodies with bactericidal activity (Bowe *et al.*, 2004). Mucosal vaccines with appropriate adjuvants have the potential advantage of preventing the initial interaction between invading pathogens and the cells of the

mucosa and thereby aiding in the elimination of the pathogen before disease is established (Kiyono and Fukuyama, 2004).

The AB-type toxins cholera toxin (CT) from *vibrio cholerae* and heat labile enterotoxin (LT) from enterotoxigenic strains of *E. coli* are potent mucosal adjuvants but can have deleterious effects *in vivo* (Williams *et al.*, 1999). Nasal co-administration of CT or LT with antigen induces antigen-specific mucosal and systemic antibody responses (Takahashi *et al.*, 1996). CT also promotes a Th2-type response against the co-administered antigens (Lavelle *et al.*, 2003), while LT promotes the generation of Th1 and Th2 type cells (Bowe *et al.*, 2004). There have been safety concerns regarding the use of certain pathogen-derived molecules as nasal adjuvants because of their binding to the GM1 ganglioside receptor an trafficking to the brain via the olfactory nerve and causing inflammation and facial paralysis (Bourguignon *et al.*, 1999, van Ginkel *et al.*, 2000, Zurbriggen *et al.*, 2003). However, mutants of CT and LT, constructed to reduce the toxic affects of the molecules, have been shown to retain immunomodulatory activity and have potential for use as nasal vaccine adjuvants (Baudner *et al.*, 2003, Giuliani *et al.*, 1998, Pizza *et al.*, 2001). The safety and efficacy of these molecules as vaccine adjuvants has been reported (Peppoloni *et al.*, 2003), however, other reports indicate that they may still traffic to the nervous system via the olfactory bulb (Eriksson *et al.*, 2004). In addition to adjuvants, the use of delivery systems to increase antigen uptake across the epithelium increase the efficacy of mucosal vaccine formulations (Ryan *et al.*, 2001). Chitosan, a naturally derived polysaccharide, and has been shown to promote transmucosal absorption of antigens and to enhance systemic and mucosal antibody responses to nasally delivered antigens (van der Lubben *et al.*, 2001).

Given that the adjuvanticity of CyaA for a co-administered bystander antigen has been demonstrated in previous chapters and that CyaA is essential for successful mucosal colonisation by *B. pertussis* it was of interest to know if CyaA could modulate the immune response when delivered mucosally, what type of response was generated and if CyaA had potential as a mucosal vaccine adjuvant. Therefore, in this study, the mucosal adjuvant activity of CyaA was investigated. CyaA co-administered with the by-stander antigen KLH via the nasal route generated both local and systemic immune

responses against the antigen. CyaA enhanced the induction of antigen-specific IL-10 producing regulatory type-1 T cells as well as IL-10, IL-5 and IL-4 producing Th2 cells. The cytokine profile of these cells was confirmed by the generation of T cell lines, T cell clones and by intracellular cytokine staining. CyaA enhanced antigen-specific IgG1 and promoted the production of antigen specific IgA in the lung. The adjuvanticity of CyaA was not confined to local immunity, and an increase in systemic antigen-specific IgG was detected in mice immunised with CyaA and KLH which was again polarized to the IgG1 sub-class compared to mice immunised with antigen alone. NadA is a novel candidate molecule for vaccination against *Neisseria meningitides* infection and has been shown to be effective at inducing bactericidal antibodies and cellular immunity when immunised nasally with LT and its derivatives as adjuvants (Bowe *et al.*, 2004, Pizza *et al.*, 2000). The adjuvanticity of CyaA for NadA when delivered nasally was investigated and it was found that CyaA promotes increased production of local NadA-specific IgA compared to mice immunised with NadA alone. In addition to modulation of local immunity, CyaA also promoted the generation of systemic NadA specific IgG, with the IgG1 sub-class increased more than IgG2a. The ability of chitosan as a delivery system to improve the adjuvanticity of CyaA was investigated, but no improvement was observed. These results demonstrate for the first time that CyaA directly modulates the immune response at the respiratory mucosa and that CyaA is an effective mucosal adjuvant, driving the generation of local and systemic immunity against co-administered antigens and as such, is a novel candidate adjuvant for mucosal vaccines.

6.2 Results

6.2.1 Nasal immunisation of antigen with CyaA increases cell recruitment to the draining lymphnodes and increased antigen-specific cell proliferation.

To investigate if CyaA has adjuvant properties when delivered by a mucosal route, mice were immunised nasally with KLH (5 μg) alone or with CyaA (2 μg) and boosted twice, 21 and 40 days later. 14 days after the final boost, the mice were sacrificed, the superior cervical lymph nodes recovered and a single cell suspension prepared. A total mononuclear cell count was performed on all samples. Mice immunised with KLH alone had higher numbers of cells in the lymph nodes when compared to mice immunised with PBS (Fig. 6.1A). A further increase in superior cervical lymph node cellularity was found in mice immunized with KLH in the presence of CyaA. Lymph node cells from mice immunised with KLH in the presence of CyaA demonstrated significant antigen-specific proliferation compared to mice that were immunised with antigen alone (Fig 6.1B). This demonstrates that CyaA promotes cellular recruitment to the draining lymph nodes of mice immunised via a mucosal route. In addition, antigen-specific cells are induced in the draining lymph nodes of these mice.

6.2.2 CyaA induces Th2 and Tr1 cells specific for a nasally co-administered antigen.

To further investigate the adjuvanticity of CyaA when delivered by a mucosal route, lymph node cells recovered from mice 14 days after 3 nasal immunisations with KLH (5 μg) alone or with CyaA (2 μg) were re-stimulated *in vitro* with antigen (KLH 2-50 μg/ml). Three days later supernatants were removed and IFN-γ, IL-4, IL-5 and IL-10 production was measured by ELISA. Mice immunised with KLH alone demonstrated a weak cellular immune response; with low concentrations of antigen-specific IL-5 but no significant IFN-γ, IL-4 or IL-10 (Fig. 6.2). In contrast, the lymph node cells from mice immunised nasally with KLH in the presence of CyaA exhibited antigen-specific IL-4, IL-5 and IL-10 production. No IFN-γ production was detected after restimulation of cells from mice immunized with KLH and CyaA. Lymph node cells from all groups

produced IL-4, IL-5, IL-10 and IFN-γ in response to the polyclonal stimuli, PMA and anti-CD3.

The cytokine profile of antigen-specific T cells detected after immunisation with KLH in the presence of CyaA by a mucosal route was similar to that observed after immunisation s.c. in the footpad (chapter 4), and suggested that CyaA promoted the development of antigen-specific Th2 and Tr1 cells at a mucosal surface. In order to confirm this, KLH-specific $CD4^+$ T cell lines and clones were generated from lymph node cells of mice immunised with KLH in the presence of CyaA. Each of the three T cell lines shown in Fig. 6.3 secreted high levels of IL-10 and IL-5, with two secreting IL-4 and all three secreting low levels to undetectable levels of IFN-γ. One of these T cell lines (8.1), which secreted IL-10, IL-5 and IL-4, with very low levels of IFN-γ was cloned by limiting dilution and the cytokine profiles of the resultant T cell clones was examined. These KLH-specific T cell clones secreted IL-10 and IL-5 or IL-10, IL-5 and IL-4, characteristic of Tr1 and Th2 type cells respectively (Fig. 6.3B). IFN-γ was at background levels in supernatants from all of the T cell clones. In addition, cytokine production by T cell line 8.1 was analysed by intracellular cytokine staining (Fig. 6.3C). It was found that 42% of the T cells tested produced both IL-4, characteristic of Th2 cells. However, 18% of the T cells produced IL-10 in the absence of any IL-4, a profile characteristic of Tr1 type cells. These findings demonstrate that CyaA is an effective mucosal adjuvant that promotes the generation of antigen specific $CD4^+$ Th2 and Tr1 cells.

6.2.3 CyaA enhances local and systemic antibodies against a nasally co-administered antigen.

To further elucidate the immune response generated in response to an antigen delivered nasally with CyaA, antibody responses were examined in serum and lungs of BALB/c mice that were nasally immunised with KLH alone or with CyaA. These mice were boosted 21 and 40 days later and lungs and blood collected after a further 14 days. IgA, IgG1 and IgG2a in the lungs and IgG1 and IgG2a in the serum was determined by ELISA. Nasal immunisation with KLH alone resulted in a modest induction of KLH specific IgA in the lungs (Fig. 6.4A). Co-administration of CyaA with KLH augmented

the production of local IgA following nasal immunisation. Nasal immunisation with KLH alone also induced a low titre of antigen-specific local IgG in the lungs. This was increased following delivery of KLH with CyaA via the nasal route. An examination of the subtypes revealed that antigen-specific IgG1, but not IgG2a, was significantly enhanced in these mice compared with mice that received KLH alone (Fig. 6.4B).

Nasal immunisation with KLH alone, resulted in a strong systemic antibody response against KLH (Fig. 6.4C). However, KLH-specific IgG titres were further increased with the inclusion of CyaA with the antigen. Significantly higher titres of antigen-specific IgG1 were detected in mice immunised with KLH and CyaA when compared with mice immunised with antigen alone, while IgG2a was not significantly different. These results are in agreement with those presented by the Coote laboratory at the 7th International Symposium on Pertussis (Orr *et al.*, 2002). These data demonstrate that CyaA is an effective adjuvant when delivered by the mucosal route, augmenting antigen specific immunity systemically and at the site of immunisation.

6.2.4 CyaA promotes immune responses against a nasally delivered candidate *Neisseria meningitidis* serogroup B vaccine antigen.

To investigate if the adjuvant properties of CyaA could be applied to a candidate vaccine antigen, the ability of CyaA to promote the generation of local immunity to NadA was assessed. NadA is a surface antigen from *N. meningitidis* expressed in all hyper-virulent species, against which a strong immune response can be elicited when delivered as a mucosal vaccine with mutants of LT as adjuvants (Bowe *et al.*, 2004). BALB/c mice were immunised nasally with NadA (5 μg) alone, or with CyaA (2 μg) on day 0 and were boosted 21 and 35 days later. After a further 10 days mice were sacrificed and lung homogenate was assayed for NadA-specific IgA and serum was assayed for IgG1 and IgG2a by ELISA. Nasal immunization with NadA alone stimulated low but detectable levels of antigen-specific IgA (Fig. 6.5A). This response was augmented when NadA was delivered with CyaA. Co-immunisation of NadA with CyaA resulted in a significant increase in antigen specific IgG1 and a lesser increase in IgG2a (Fig. 6.5B). These results demonstrate that CyaA functions as an adjuvant for a

pathogen derived antigen, increasing both local and systemic immunity, and confirms that CyaA has potential as a vaccine adjuvant when delivered by the mucosal route.

6.2.5 Chitosan does not improve the antigen-specific antibody immune response to NadA delivered nasally with CyaA.

Having demonstrated that CyaA has mucosal adjuvant activity, I addressed the hypothesis that the systemic immune response generated by CyaA against a nasally co-administered antigen could be enhanced by formulation with the mucosal delivery system, chitosan. BALB/c mice were immunised nasally with NadA (10 μg) alone or with CyaA (1 μg) in the presence or absence of chitosan (25 μg). Mice were boosted 21 and 35 days later and sacrificed after a further 10 days. Serum was recovered and tested for antigen-specific IgG. Immunisation with 10 μg of NadA alone induced the production of significant amounts antigen-specific IgG1 and IgG2a (Fig. 6.6). Co-administration of NadA with CyaA increased the antigen-specific antibody production and when the subclasses were examined it was found that IgG1 was the dominant subclass. However, antigen-specific antibody production was not enhanced by the inclusion of chitosan in the formulation. These results demonstrate that chitosan as a carrier molecule does not improve the systemic antigen specific antibody response generate by nasal immunisation with NadA in the presence of CyaA.

6.3 Discussion

Traditional vaccines developed in the early part of the last century were based on whole live attenuated or inactivated viruses or bacterial cells. After safety concerns were highlighted, the development and use of sub-unit vaccines became a priority. However these sub-unit vaccines are usually less immunogenic than heat-killed or attenuated vaccines, and necessitate the use of an adjuvant to enhance their immunogenicity (Marciani, 2003, O'Hagan and Valiante, 2003). Unfortunately most adjuvants licensed for human use do not generate strong cellular immune responses, needed for immunity against important pathogens such as HIV and TB and are not suitable for delivery by the mucosal route (Holmgren and Czerkinsky, 2005, Robinson and Amara, 2005). This has prompted research into the development of new safe and potent adjuvants capable of promoting cellular immunity at the mucosal surfaces. In addition, there is an increasing awareness that the underlying immunostimulatory effects of an adjuvant need to be characterised and understood. As well as the nature of both the antigen and adjuvant, the route of immunisation must also be considered in the design of successful vaccines (Holmgren and Czerkinsky, 2005). The majority of modern vaccines are delivered by intramuscular injection, which ensures that the entire dose is administered intact into the muscle tissue. Mucosally delivered vaccines remove the need for injections administered by medically trained personnel, eliminate concerns over reuse or disposal of contaminated needles and help to improve patient compliance and vaccine uptake. Alum is one of the few adjuvants licensed for human use and the most widely used. However, despite its long history and widely accepted success, there are some concerns about its safety, although there are no suggestions that its use should be discontinued. Alum is not used in mucosal vaccines and focus instead lies on the development of novel adjuvants for mucosal delivery, in particular, immunostimulatory molecules whose role in stimulating immunity is known and understood. To this end the use of synthetic and pathogen derived molecules that bind TLRs and other PRRs to boost the immune response to a vaccine is the focus of much research. CpG-ODN, which bind TLR9 (Harandi and Holmgren, 2004), and CT, LT and their derivatives, which bind GM1 gangliosides on the surface of cells have been shown to have great potential as mucosal adjuvants (Baudner *et al.*, 2003, Giuliani *et al.*, 1998, Pizza *et al.*,

2001). However, only a portion of the antigen in vaccines delivered by the mucosal route will be transported across the epithelium, this is a particular issue with orally delivered vaccines (Holmgren and Czerkinsky, 2005, Kiyono and Fukuyama, 2004). To address this, the use of delivery systems, such as chitosan, microspheres and liposomes, is being investigated and each have been shown to aid the transport of vaccines and adjuvants across the mucosa increasing the potency of the immune response (de Jonge *et al.*, 2004, Felnerova *et al.*, 2004, Vajdy and O'Hagan, 2001, van der Lubben *et al.*, 2001).

As well as having potential as an immunotherapeautic agent, CyaA is a potent parenteral adjuvant that enhances antigen-specific Th2, Tr1 responses and IgG1 polarised antibody responses. This study examined the ability of CyaA to act as a mucosal adjuvant when co-administered with antigens by the nasal route. The results show that CyaA promotes the recruitment of cells to the local draining lymph nodes after nasal immunisation, a key step in the initiation of adaptive immunity. Activation of DC and their homing to the lymph nodes is a critical step in the initiation of an immune response, and is necessary for the priming, polarisation and proliferation of antigen-specific T-cells. The data shown previously in chapter 4 demonstrates that CyaA modulates the activation of innate immune cells and the maturation of DC. Nasal delivery of KLH with CyaA generated antigen-specific proliferative responses in lymph node cells and induced a Th2/Tr1 response in mice. $CD4^+$ T cells from mice immunised with KLH and CyaA produced IL-10, IL-5 and IL-4 when re-stimulated *in vitro* with antigen and T cell lines cultured from these cells demonstrated a bias towards IL-10 production. Furthermore, T cell clones generated from these lines demonstrated the presence of Tr1 type cells producing IL-10 and IL-5 in the absence of IL-4 and also Th2 type cells that secreted IL-10, IL-5 and IL-4.The induction of distinct populations of Tr1 and Th2 cells was confirmed by intracellular cytokine staining of the polyclonal T cell lines. These results demonstrate that CyaA has immunomodulatory effects at the mucosa and are consistent with the observations that parenteral delivery of CyaA induces antigen-specific anti-inflammatory and regulatory cytokine production. The immunomodulatory effect of CyaA at the mucosa was further demonstrated by an increase in the production of local antigen-specific IgA and IgG1 in the lungs of mice

immunised with antigen and CyaA. In addition to stimulating immunity at the site of immunisation, an effective vaccine must also induce systemic immunity against the pathogen. Mice immunised nasally with KLH and CyaA had significantly higher titres of systemic antigen-specific IgG1 than in mice immunised with KLH alone. these findings demonstrates that CyaA is a potent adjuvant when delivered by a mucosal route.

NadA is a surface molecule expressed on all hypervirulant strains of *N. meningitidis* (Comanducci *et al.*, 2002) and is a promising vaccine candidate antigen for the development of a vaccine against serogroup B meningococcous (Bowe *et al.*, 2004). CyaA enhanced immune responses to NadA when co-administered nasally. Immunisation of mice with NadA in the presence of CyaA induced stronger local and systemic antibody responses than immunisation with NadA alone. Carrier molecules and delivery systems are used to enhance the delivery and immunogenicity of molecules delivered by the mucosal route. Chitosan, a polysaccharide, has been shown to be an effective carrier molecule for bacterial derived molecules (van der Lubben *et al.*, 2001). The addition of chitosan to the formulation when mice were immunised with NadA and CyaA did not enhance the NadA-specific immune response when compared with mice immunised with NadA and CyaA without chitosan. While not as pronounced as the adjuvanticity for KLH, these results demonstrate that CyaA does promote antigen specific immunity to a bacterially derived antigen. Similar to CT and LT, the adjuvanticity of CyaA is not limited to a model antigen but can promote the immune response to a novel candidate vaccine antigen. However, as CyaA has been shown to induce lysis of cells, concerns regarding the use of a potentially toxic molecule, similar to concerns over the use of CT and LT as nasal adjuvants, would have to be addressed. The use of non-toxic derivatives of CT and LT have been reported and show that while lacking cytotoxicity they retain the ability to modulate the immune response and act as vaccine adjuvants (Baudner *et al.*, 2003, Giuliani *et al.*, 1998, Pizza *et al.*, 2001). However, there remains some concern over their use as LT or CT (Eriksson *et al.*, 2004) have been shone to still traffic to the nervous system through the olfactory bulb following nasal immunisation. This trafficking is thought to be mediated by binding to GM1-gangleoside on the surface of cells. CyaA, however, does not bind GM1-

ganglioside, instead targeting cells of the innate immune system by binding CD11b/CD18 and may not traffic to the nervous system. This remains to be addressed. Preliminary work in the laboratory indicates that CT and its derivatives are several hundred times more potent (μg/μg) than CyaA in terms of its adjuvant effects. CyaA does not cause the immune pathologies sometimes associated with CT, even at low amounts. CyaA can therefore be used at higher doses and is a more attractive candidate therapeutic molecule for this reason. Furthermore, the development of non-toxic derivatives of CyaA capable of driving an immune response to a nasally co-administered antigen would be positive step in the clinical use of CyaA as an adjuvant and immunotherapeutic molecule.

These results also provide some insight into the role played by CyaA plays during the course of *B. pertussis* infection, and how CyaA may modulate immune response at the site of infection. Studies in a mouse model of infection have shown that CyaA is required for the virulence of *B. pertussis* (Goodwin and Weiss, 1990), and the absence of CyaA dramatically reduces the pathological effects of *B. pertussis*, reducing the numbers of bacteria in the lung (Gross *et al.*, 1992, Weiss *et al.*, 1984) and abolishing inflammatory cell recruitment (Harvill *et al.*, 1999, Khelef *et al.*, 1994). Indeed, CyaA inhibits anti-bacterial functions such as chemotaxis, phagocytosis and superoxide generation in host immune cells (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987, Weingart *et al.*, 2000). In addition CyaA has been shown to cause lysis of a variety of cells including RBC, macrophages, DC and T cells (chapter 4 and (Lee *et al.*, 1999, Westrop *et al.*, 1997), and to induce apoptosis of alveolar macrophages (Gueirard *et al.*, 1998, Khelef and Guiso, 1995, Khelef *et al.*, 1993). Thus CyaA subverts the immune response against *B. pertussis* thereby facilitating the persistence of the bacteria and the establishment of disease. The data shown here adds to the knowledge of how *B.pertussis* regulates the host immune response during infection through t he production of CyaA. In addition to killing cells and interfering with cellular functions at the site of *B. pertussis* infection, CyaA may synergise with other *B. pertussis* factors to induce IL-10 secretion by innate immune cells and promote Tr1 cell differentiation. Innate IL-10 and Tr1 cells have been shown to down regulate inflammatory cell function and suppress IFN-γ secretion from Th1 cells (Lavelle *et al.*,

2003), both of which are important in the clearance of *B. pertussis* infection from the lung (Mahon *et al.*, 1997, Mills *et al.*, 1993). *B. pertussis* specific IL-10 secreting T cells have been detected following *B. pertussis* infection (Higgins *et al.*, 2003). The induction of these IL-10 secreting T cells during *B. pertussis* infection is dependent on a functional TLR4 receptor. This is consistent with the requirement of CyaA for TLR co-stimulation to effectively modulate immature DC into a phenotype that promotes the induction of Tr1 cells. Although *B. pertussis* infection of TLR4-defective mice is cleared in a similar time frame to that seen in wild type mice, the bacterial burden in TLR4-defective mice is significantly increased compared with wildtype mice (Higgins *et al.*, 2003). Mice that lack TLR4 signalling and IL-10 secreting T cells have more severe inflammation and immune mediated pathology in the *B. pertussis* infected lungs when compared to an infection in wildtype mice. This uncontrolled immune response in TLR-4-defective mice demonstrates that *B. pertussis* regulates the immune response in the lung to promote its survival. In fact, it has been shown that FHA, an important adhesin and virulence factor of *B. pertussis* is necessary for optimal colonisation of the lungs (Relman *et al.*, 1989), but also promotes the generation of Tr1 type cells (McGuirk *et al.*, 2002). The data presented here demonstrates that CyaA can regulate immune responses at the mucosal surface and suggests that CyaA may play a role in regulating immunity to *B. pertussis* during the course of an infection, by promoting innate IL-10 production and the generation of antigen-specific Tr1 cells.

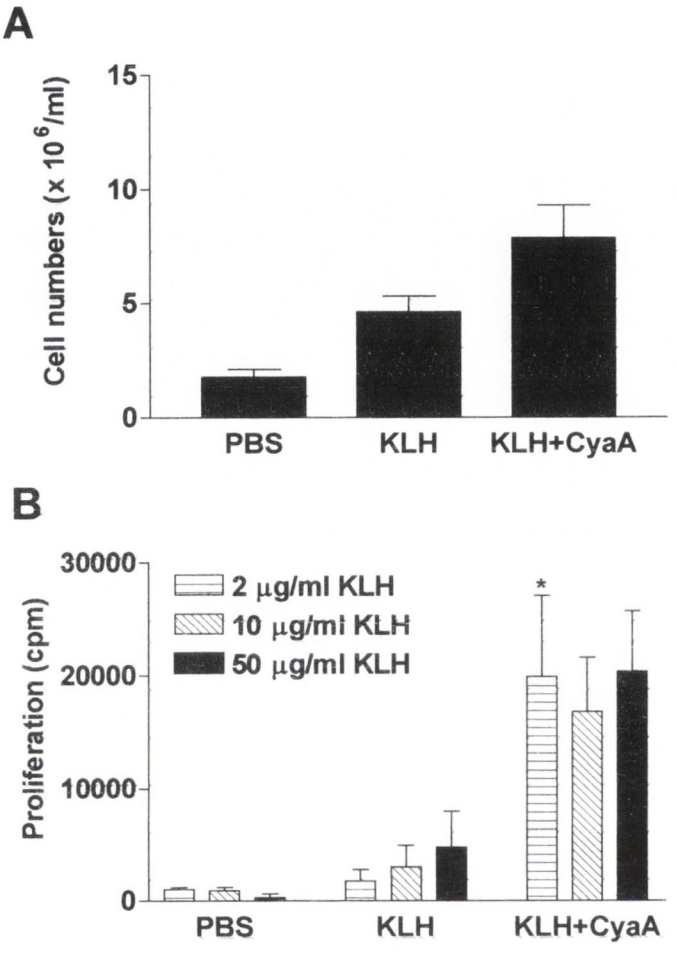

Figure 6.1 Nasal immunisation of antigen with CyaA increased cell recruitment to the draining lymph nodes and increased antigen specific proliferation response.

BALB/c mice were immunised nasally with PBS, KLH (10 μg/), or KLH with CyaA (2 μg) on day 0. Mice were boosted at 21 and 40 days and sacrificed 14 days after the last immunisation. Superior cervical lymph node cells were prepared and stimulated with KLH (2-50 μg/ml). Proliferation was assayed on day 4 by [3H]thymidine incorporation. Results represent means (error bars, SD) of five mice per group and are representative of two experiments. * $P < 0.05$ KLH versus KLH+CyaA

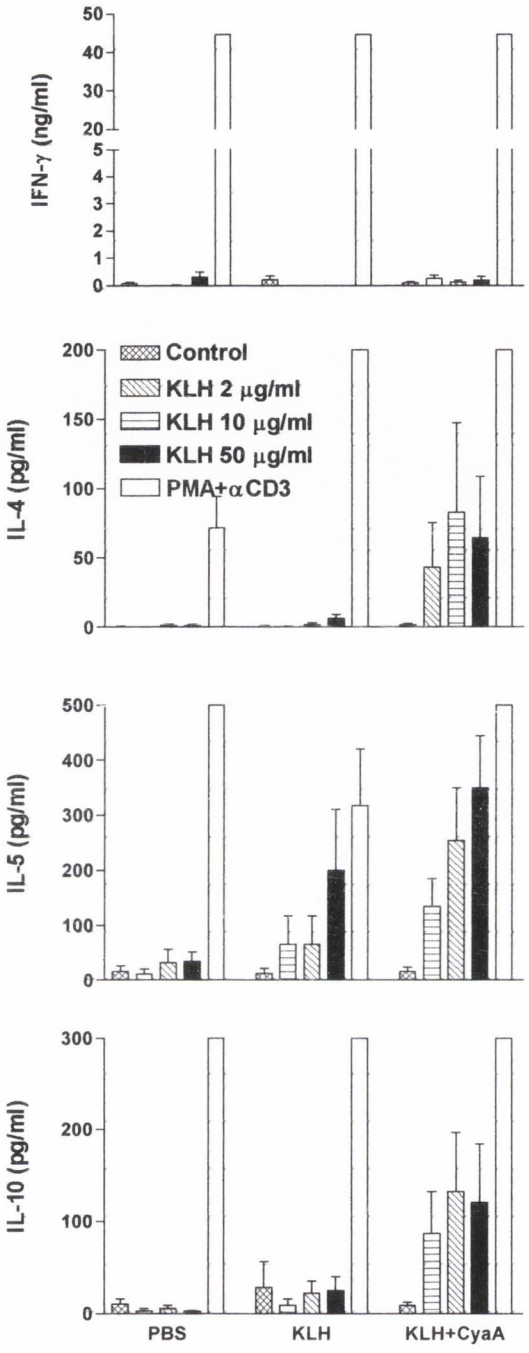

Figure 6.2 CyaA induces Th2/Tr1 responses to a nasally co-administered antigen.
BALB/c mice were immunised nasally as described in Fig. 6.1. Superior cervical lymph node cells were prepared and stimulated with KLH (2-50 μg/ml), PMA and anti-CD3 or medium only. After 3 days, supernatants were tested for IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Results represent means (error bars, SD) of five mice per group and are representative of two experiments.

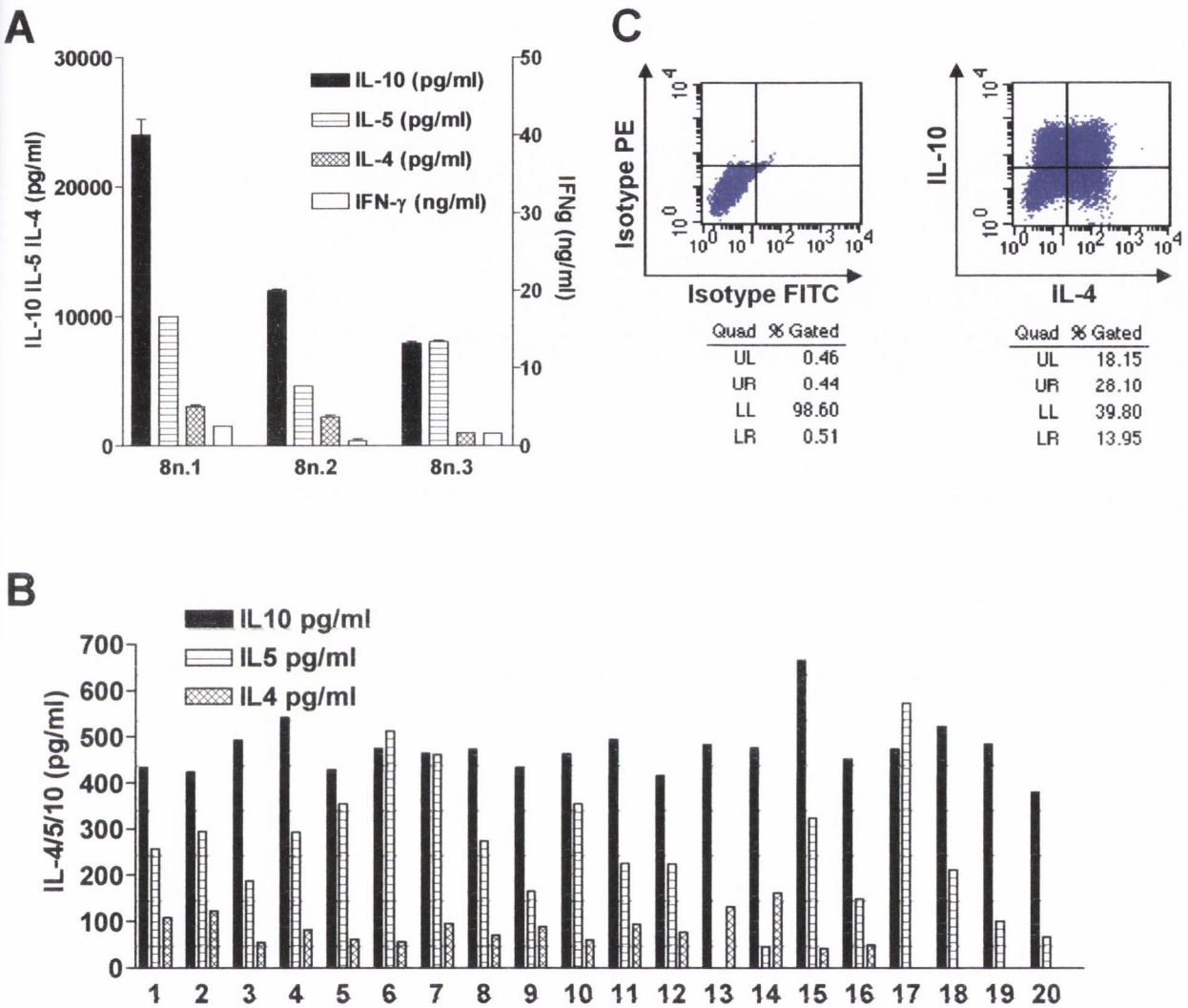

Figure 6.3 KLH-specific Th2 and Tr1 cells generated from mice nasally immunised with KLH in the presence of CyaA.

(A) $CD4^+$ T cell lines were generated from the superior cervical lymph nodes of 3 individual mice immunised with KLH and CyaA. (B) T cell line 8.1 was cloned by limiting dilution. T cells lines or clones were stimulated with KLH (50 μg/ml) in the presence of autologous APC and cytokine concentrations were measured by ELISA after three days. Results are cytokine profiles for lines or clones cultured in individual wells of 96 or 24 well plates and are representative of 2 different assays. IFN-γ was at background levels in each of the T cell clones. (C) T cell line 8.1 was assayed for intracellular IL-10 and IL-4 (right panel) and compared with the appropriate isotype controls (left panel). Results are given as percentages of $CD4^+$ T cells positive for the cytokines indicated and are representative of two experiments.

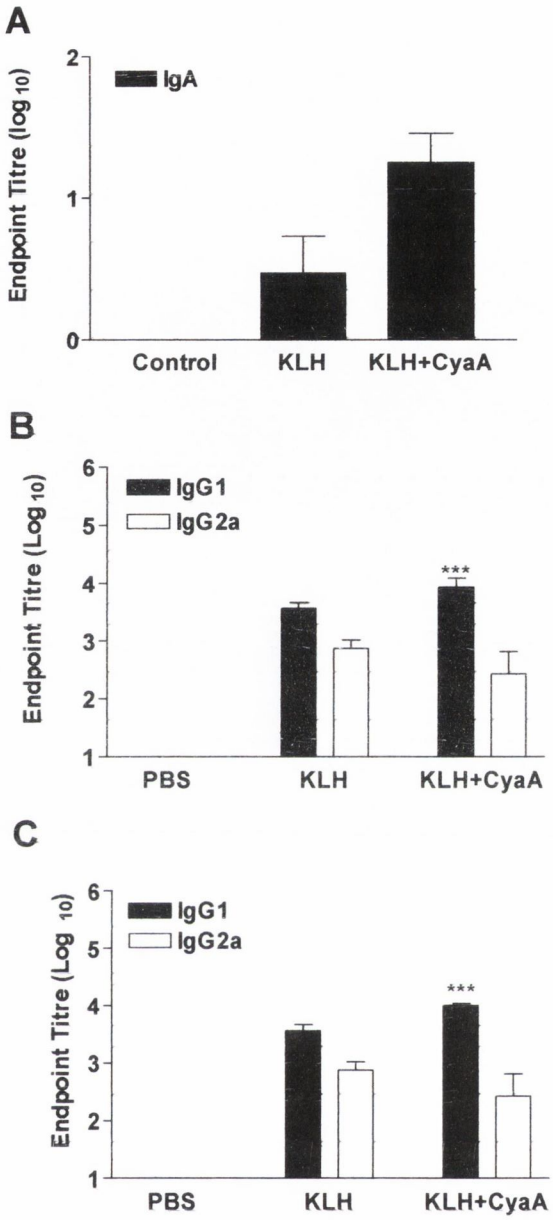

Figure 6.4 Nasal immunisation of KLH with CyaA promotes antigen-specific IgA and IgG1 antibodies in the lungs and IgG1 antibodies in the serum.

Mice were immunised nasally with PBS, KLH (10 μg) alone, or with CyaA (2 μg) and boosted 21 and 40 days later and sacrificed after a further 14 days. Lung homogenate was assayed for KLH-specific IgA (A), IgG1 and IgG2a (B) and serum was assayed for KLH-specific IgG1 and IgG2a (C) by ELISA. Results are mean (+ SD) titres for 5 mice per group and are representative of 2 experiments. *** $P < 0.001$ KLH versus KLH+CyaA.

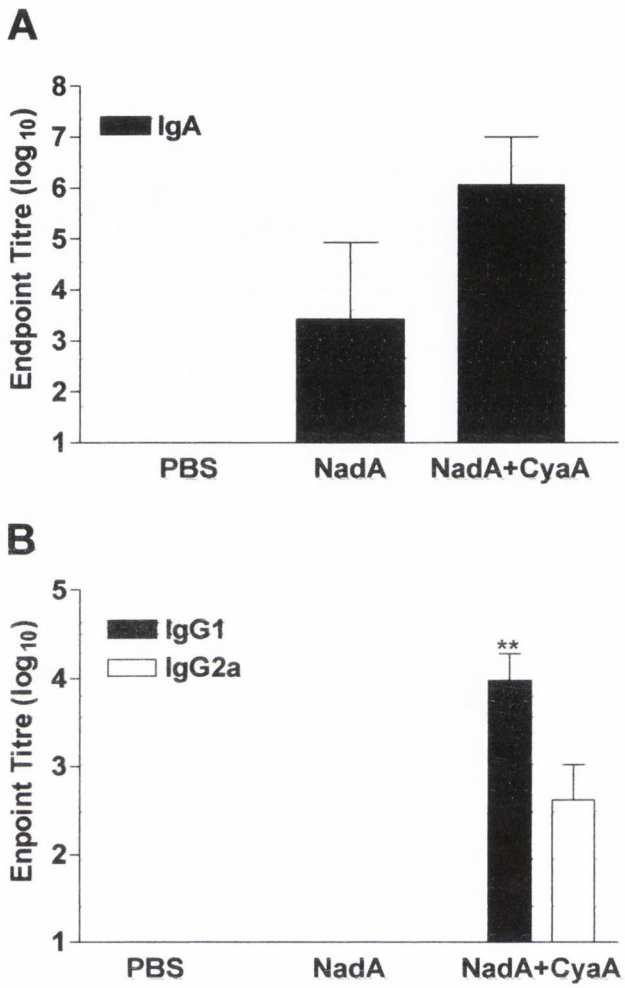

Figure 6.5 CyaA enhances lung IgA and serum IgG1 to nasally co-administered NadA.
Mice were immunised nasally with PBS, NadA (5 μg), or NadA with CyaA (2 μg), were boosted 21 and 35 days later and sacrificed after a further 10 days. Lungs and serum were recovered. (A) Lung homogenate was assayed for NadA-specific IgA and (B) serum for IgG1 and IgG2a by ELISA. Results are mean (+ SD) titres for 5 mice per group. ** $P < 0.01$ NadA versus NadA+CyaA.

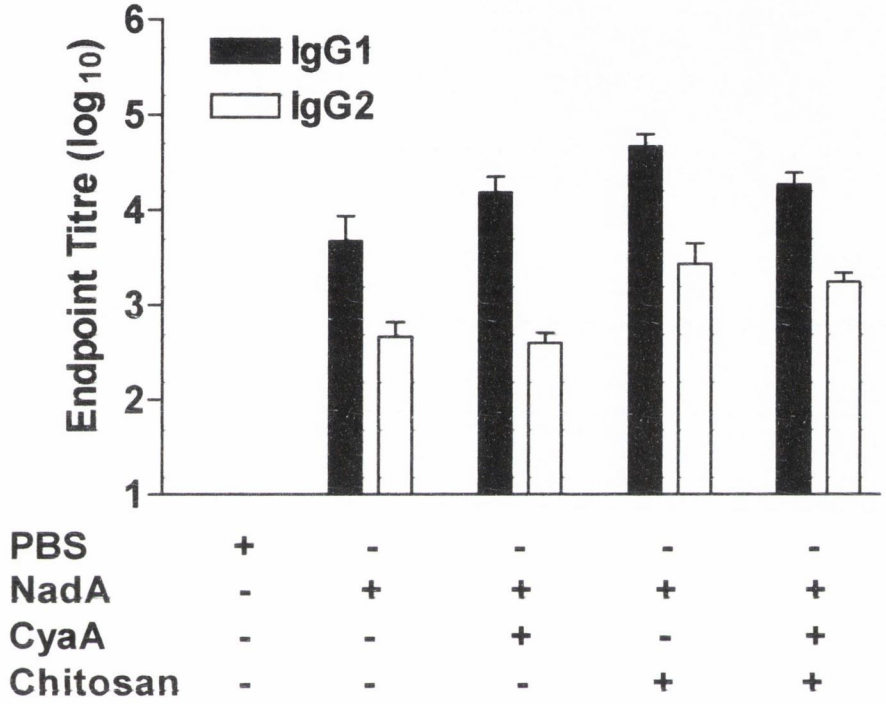

Figure 6.6 Chitosan does not improve antigen specific serum IgG responses modulated by CyaA to a nasally co-administered *N. meningitis* antigen.
Mice were immunised nasally with PBS, NadA (10 μg) alone or with CyaA (1 μg) in the presense or absence of chitosan (25 μg), and were boosted 21 and 35 days later and sacrificed after a further 10 days. Serum was collected and assayed for NadA-specific IgG1 and IgG2a by ELISA. Results are mean (+ SD) titres for 5 mice per group.

Chapter 7

TRANSCRIPTIONAL ANALYSIS OF THE MODULATION OF THE INNATE IMMUNE RESPONSE BY CYAA

7.1 Introduction

The results in chapter 4, 5 and 6 have shown that CyaA is a potent immunomodulatory molecule and acted on innate immune cells to promote the secretion of the regulatory cytokine IL-10 while suppressing TLR ligand-induced production of the inflammatory cytokines, IL-12p70 and TNF-α. In addition, CyaA promoted the generation of IL-10-secreting Tr1 cells *in vivo*. CyaA specifically targets cells of the innate immune system that express CD11b/CD18 (Guermonprez *et al.*, 2001) and plays an important role in establishing and maintaining *B. pertussis* infection (Goodwin and Weiss, 1990). CyaA also inhibits anti-bacterial functions of innate cells, including superoxide generation, phagocytosis and chemotaxis (Friedman *et al.*, 1987, Khelef *et al.*, 1994, Narayanan *et al.*, 2002, Pearson *et al.*, 1987). In addition, CyaA causes lysis and cell death in macrophages (Khelef *et al.*, 1994). The results in the previous chapters demonstrated that formation of intracellular cAMP by CyaA might mediate a significant proportion of its immunomodulatory activities. However, the precise mechanisms by which CyaA modulates the activity of innate cells and promotes the survival of *B. pertussis* in the host is not fully understood.

Macrophages, like DC, play an important role in the host's innate and adaptive responses against pathogens. Macrophages recognise pathogens through PRRs and phagocytose and destroy pathogens and also act as APC. Cytokines produced by macrophages are important in the activation and polarisation of the adaptive T cells response against the pathogen. Pathogens have evolved strategies to circumvent macrophage defence mechanisms in order to promote their survival. *Salmonella* spp. induce apoptosis (Hersh *et al.*, 1999), while *Yersinia* delivers inhibitory type III system effectors which regulate innate immune cell functions (Sauvonnet *et al.*, 2002) and *B. pertussis* induces regulatory T cells which suppress protective Th1 cells (McGuirk *et al.*, 2002).

A greater understanding of the interactions between pathogen-derived molecules and cells of the immune system and the subsequent activation of intracellular pathways in these cells would lead to a greater understanding of the defence strategies of the host and the evasion strategies of the pathogen. Microarray technology allows the large-scale simultaneous analysis of gene expression (Stears *et al.*, 2003). Many studies

investigating the interplay between pathogens and hosts have provided insight into the mechanisms of host defence and the strategies employed by pathogens to subvert immune responses of the host in order to promote their survival. Microarray technology has been useful in understanding how innate and adaptive immune cells respond to infection and how the host responds to different pathogens and to different strains of a single pathogen (Blumenthal *et al.*, 2005, Imai *et al.*, 2005, Leong *et al.*, 2005). In addition, analysis of the transcriptome has also been used to further understanding of how specific pathogen-derived molecules and virulence factors are capable of modulating the innate and adaptive immune responces (Detweiler *et al.*, 2001, Hedges *et al.*, 2005, Huang *et al.*, 2001).

CyaA modulates TLR-induced responses in innate cells and is recognised by the host in the context of many other virulence factors produced by *B. pertussis*. CyaA also synergises with TLR ligands to promote the generation of anti-inflammatory responses in innate cells. In this chapter microarray technology was used to investigate how CyaA modulated gene expression in macrophage responses. These responses were compared with those observed with the TLR9 ligand CpG. Furthermore, responses of macrophages to co-treatment with CyaA and CpG was also investigated. Immune responses to pathogens and pathogen-derived molecules varies over time. In addition, the expression of genes in response to stimuli can also change with time. The magnitude of the immune response generated by CyaA altered with time (chapter 4), therefore, the modulation of macrophage gene expression was investigated by mircoarray technology at early and late timepoints (0.5 and 6 hours).

CyaA was found to synergise with CpG to upregulate anti-inflammatory genes including CD69, IL-1Ra, IL-1Rβ and SOCS3. In addition, pro-inflammatory genes, such as TLR4, IL-7r and TRAF1, were down-regulated by CyaA. Although pro-inflammatory genes, such as IL-1β, were found to be upregulated by CyaA, it was also demonstrated that genes for antagonists to pro-inflammatory molecules, such as IL-1Ra and IL-1Rβ, were upregulated by CyaA, indicating a negative regulation of pro-inflammatory responses. CyaA alone modulated gene expression, and suppressed a large number of genes 0.5 h after treatment while CyaA upregulated a large number of genes 6 h after treatment. It was also found that the sets of genes modulated by CyaA

varied over time, indicating a temporal regulation of gene expression induced by CyaA. These data demonstrate that CyaA activates a complex pattern of gene expression that varies over time and is characterised by the activation of anti-inflammatory genes and the suppression of pro-inflammatory genes. These results indicate that CyaA plays a complex role during *B. pertussis* modulating immune responses that promote survival of the bacteria in the host.

7.2 Results

7.2.1 Treatment of cells and isolation of RNA

To investigate the effects of CyaA on the modulation of immune responses of innate cells at a transcriptional level the murine J774 macrophage cell line was stimulated with CyaA (1 μg/ml) or CpG (10 μg/ml), alone or together or untreated as a control and RNA analysed by microarray. While an investigation of the modulation of gene transcription in DC by CyaA would have proved interesting in the context of the previous chapters it was not possible to obtain a pure enough population of naïve cells, as few as 1% contaminating cells can generate spurious results in microarray experiments (Stears *et al.*, 2003). Unfortunately no suitable naïve DC cell line was available. However, macrophages play an important role in immune responses and the previous results have demonstrated that CyaA modulates macrophage cytokine production in a similar fashion to that of DC. The J774 macrophage cell line is 100% homogeneous and naïve. For these reasons the modulation of gene expression in J774 macrophages by CyaA was examined. Experiments were performed in the presence of polymyxin-B to negate the effects of any residual LPS present in the protein preparations. Three biological replicates of $1x10^6$ cells were treated with each stimulus and supernatants were collected and RNA purified from cells at 0.5, 2 and 6 hours following treatment and pooled to reduce noise and spurious results in microarray experiments. Two independent experiments were performed and the results were found to be consistent, the results for one experiment are presented here. IL-10 and TNF-α production was tested by ELISA (Fig. 7.1). Consistent with previous results in chapters 4 and 5, CyaA synergised with the TLR9 ligand CpG to promote the secretion of IL-10 from macrophages. In addition, CyaA suppressed CpG-stimulated production of the pro-inflammatory cytokine, TNF-α. These effects on cytokine secretion were clearly observed in cells treated for 6 h, but at much lower levels at 2 h following treatment. Treatment with CyaA alone did not stimulate IL-10 or TNF-α production at any timepoint examined. Furthermore, treatment with CyaA and CpG, either alone or together, did not stimulate production of IL-10 or TNF-α secretion at 0.5 h. These results demonstrate that the macrophage cells were responding to CyaA and CpG in

accordance with previous results and were suitable for transcriptomic analysis and RNA was extracted.

Good quality pure RNA is required to perform analysis of experimental samples by microarray technology. To confirm purity and quality of the purified RNA, each sample was analysed on a BioAnalyiser 2100 machine, which combines microfluidics and spectrophotometry to assess the quantity and quality of RNA samples. All samples were found to have high quality RNA, were not degraded and had no contamination with genomic DNA (Fig. 7.2). Distinct peaks corresponding to 18S and 28S ribosomal RNA were identified in all samples. To investigate how CyaA modulates gene transcription in innate cells at early and late timepoints, the RNA purified from macrophages following treatment with CyaA in the presence or absence of CpG for 0.5 h and 6 h was analysed by microarray using the Affymetrix commercial 430 2.0 GeneChip which allows analysis of the complete murine gene expression set.

7.2.2 Differential gene regulation by CyaA and CpG

Analysis of the mRNA expression patterns of cells treated with CyaA or CpG using the GeneChip technology allows a complete profile of all genes activated or suppressed in macrophages by treatment with these immunomodulators. Using the GeneSpring analysis software package 11560 transcripts out of the 45101 represented on the microarray were identified as being present or marginally present across all samples. This list of 11560 genes was used as the refined universal gene set from which all subsequent lists were generated to facilitate a more robust analysis.

Using a cut off point of a 3 fold change (either up- or down-regulation) it was found that treatment with CyaA modulated 120 genes following a 0.5 h treatment, while CpG modulated only 8 genes. Treatment of macrophages with CyaA and CpG for 0.5 h resulted in more than a 3 fold change in 201 genes. This demonstrated a synergistic effect of co-stimulation with CyaA and the TLR9 ligand on gene regulation of macrophages. When these data were further analysed and the genes that are upregulated by each of the treatments were compared it was found that CpG did not have a positive effect on the transcription of any gene at 0.5 h (Fig 7.3A). However,

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treatment of macrophages with CyaA alone for 0.5 h upregulated 30 genes, while treatment of macrophages with CyaA in the presence of CpG upregulated 120 genes. Only 6 common genes were upregulated by CyaA and co-treatment with CyaA and CpG (Table 7.1). Treatment with CyaA uniquely upregulated 27 genes (Table 7.2) at 0.5 h and 114 genes were uniquely upregulated by co-treatment of macrophages with CyaA and CpG (Table 7.3). This demonstrates that CyaA alone does have an effect on innate cell activation and that CyaA synergises with CpG to upregulate an almost completely different set of genes in macrophages.

When the genes whose expression was downregulated by at least 3 fold it was found that CpG downregulated 8 genes at 0.5 h (Table 7.4), none of which were downregulated by treatment with CyaA or CyaA with CpG (Fig 7.4A). CyaA downregulated 87 genes after treatment for 0.5 h and treatment with CyaA and CpG downregulated 81 genes. However, there were only 2 genes downregulated in common between CyaA and co-treatment with CyaA with CpG after 0.5 h (Table 7.5). Treatment with CyaA uniquely downregulated 85 genes at 0.5 h (Table 7.6), while treatment with CyaA and CpG resulted in the unique downregulation of 79 genes (Table 7.7). This result is consistent with the upregulation of genes by CyaA and CpG and indicates that CyaA alone modulates a different set of genes compared to co-treatment with CyaA and CpG.

Analysis of genes upregulated by CyaA and CpG 6 hours post treatment (Fig 7.3B) revealed that CpG activated 178 genes compared to CyaA treatment, which resulted in the upregulation of 334 genes. Co-treatment of macrophages with CyaA and CpG for 6 hours upregulated 346 genes. Although this is not increased above the number of genes upregulated by CyaA alone, comparison of the three gene lists demonstrates that there are 86 genes uniquely upregulated by treatment of CyaA with CpG after 6 h stimulation (Table 7.8). Treatment of macrophages with CyaA for 6 h uniquely upregulated 99 genes in macrophages (Table 7.9), while CpG treatment uniquely upregulated 62 genes (Table 7.10).

Following treatment of macrophages with CyaA and CpG for 6 hours it was found that CpG downregulated 71 genes, while CyaA treatment downregulated 65 genes (Fig. 7.4B). Co-treatment of macrophages with CyaA and CpG for 6 h resulted in

the downregulation of 168 genes, 127 of which were uniquely downregulated with this treatment (Table 7.11). CpG uniquely down regulated 57 genes following treatment for 6 h (Table 7.12) and CyaA uniquely downregulated 31 genes (Table 7.13).

These results demonstrate that modulation of gene expression in macrophages by CyaA occurs earlier than CpG and that there are very few genes commonly regulated by CyaA or CpG treatment of macrophages. In addition, co-treatment of macrophages with CyaA and CpG results in the modulation of a distinct set of genes not regulated by either treatment alone, which demonstrates the synergistic role of TLR ligands in immunomodulation by CyaA.

7.2.3 Modulation of genes by CyaA and CpG differs over time.

To investigate if modulation of gene expression by CyaA and CpG was consistent at early and late time points, the lists of genes uniquely regulated by treatment of macrophages with CyaA and CpG at 0.5 h or 6 h post treatment were compared.

Comparison of sets of the genes uniquely upregulated by co-treatment with CyaA and CpG for 0.5 h and 6h revealed only 7 genes common to both lists (Fig. 7.3C; Table 7.14), with 107 genes uniquely upregulated at 0.5 h and 79 genes at 6 h. A comparison of the genes uniquely downregulated by co-treatment of macrophages by CyaA and CpG at 0.5 h and 6 h shows that there were only 3 genes common to both sets of genes (Fig. 7.4C; Table 7.15). Co-treatment with CyaA and CpG results in the unique downregulation of 76 genes after 0.5 h while 124 genes are uniquely downregulated after 6 h. These results indicate that there is a temporal difference in the modulation of gene expression by CyaA and CpG and that there are early and late response genes activated and suppressed by co-treatment with CyaA and CpG.

A comparison of the 27 genes uniquely upregulated by CyaA at 0.5 h (Fig 7.3A) with the 99 genes uniquely upregulated by CyaA at 6 h (Fig 7.3B) reveals no common genes. Similarly, there were no common genes between the 85 genes uniquely downregulated by CyaA at 0.5 h (Fig 7.4A) and 31 genes uniquely downregulated following 6 h treatment with CyaA (Fig 7.4B). This demonstrates that treatment with CyaA alone modulates distinct sets of genes at early and late timepoints.

The 8 genes modulated by CpG treatment for 0.5 h were downregulated (Fig 7.4A; Table 7.4), although none of these genes were downregulated by CpG following 6 h of treatment. CpG modulates 249 genes following 6 h treatment compared to just these 8 genes modulated at 0.5 h. This indicates that CpG exerts its effects on gene transcription in macrophages at later timepoint compared with CyaA.

7.3 Discussion

CyaA is a potent immunomodulator produced by *B. pertussis* and is essential for colonisation of the host by the bacterium. Results presented in previous chapters have elucidated how CyaA modulates the innate and adaptive immune system. CyaA induces the secretion of the anti-inflammatory cytokine, IL-10, from innate cells in combination with a TLR ligand and suppresses the production of TLR ligand-induced inflammatory cytokines including IL-12p70 and TNF-α. CyaA also modulates surface molecule expression on DC, upregulating CD80, but downregulating the expression of the co-stimulatory molecules, CD40 and ICAM-I. This immunomodulation was dependant on the enzymatic activity of CyaA, which catalyses the accumulation of supraphysiological levels of cAMP in the cytosol of target cells. CyaA also promoted the induction of antigen-specific IL-10 secreting Th2 and Tr1 cells *in vivo*. It has been shown that CyaA binds to the CD11b/CD18 integrin on the surface of innate immune cells (Guermonprez *et al.*, 2001) and can form pore in the cell membrane and induce lysis and apoptosis (Gray *et al.*, 1998, Khelef *et al.*, 2001). While it has been demonstrated that CyaA modulates cytokine production and co-stimulatory molecule expression on DC and macrophages, it was not clear exactly how these changes were mediated within the host cell and how these effects result in immunomodulation. To better understand this a transcriptional analysis of how CyaA modulated macrophages was carried out.

The data reveal that modulation of gene transcription by CyaA is complex. CyaA increases expression of anti-inflammatory genes such as Fos, IL1rn and IL-1r2 and also suppresses the expression of pro-inflammatory genes such as TLR4 and NFAT2 are also observed. However, suppression of anti-inflammatory genes, including Tgfbr1 and CCL2, together with the activation of pro-inflammatory genes including IL-1β and EBi3 is also observed. In addition, CyaA activated both pro- and anti-apoptotic gene transcription. The data does not reveal a distinct pathway for the polarisation of the anti-inflammatory and regulatory type responses observed in immune cells treated with CyaA in previous chapters. Instead, a complex network of gene transcription is observed. It likely that the interplay of many of these genes, both pro- and anti-

inflammatory result in the activation of the immune response induced by CyaA and also the modulation of immune cell responses to TLR ligands by CyaA. To add further complexity, several of the genes whose expression is modulated by CyaA have multiple activities. For example, TRAF1 is involved in IL-1 mediated inflammatory responses and is upregulated by CyaA. However TRAF1 has also recently been shown to inhibit downstream TRIF mediated responses (Su *et al.*, 2005, Takatsuna *et al.*, 2003). The transcription factor JunB is activated by CyaA and has been shown to have positive effects on the generation of anti-inflammatory responses (Gao *et al.*, 2004). However, JunB also plays a role in the arrest of cell cycle progression (Andrecht *et al.*, 2002). The activation of genes with multiple roles makes interpretation of the data more difficult.

The importance of the induction of supraphysiological levels of cAMP by CyaA in modulating responses of innate immune cells has been demonstrated in the previous chapters. Interestingly, the transcription of cAMP response element binding protein (CREB) was not observed in these experiments. CREB is an important transcription factor and is activated in response to cAMP increases and plays a roll in cell activation and proliferation (Kruger *et al.*, 1997, Lee *et al.*, 2005a). However it is possible that CREB mediated responses are activated through phosphorylation of cytosolic CREB protein. In addition, the activation of cyclic AMP-response element modulator (CREM) is observed. CREM is an important transcription factor that activates both repressors and activators of cAMP mediated responses (Lamas *et al.*, 1996). Interestingly, the splice variant of the CREM gene which generates the transcription factor inducible cAMP early repressor (ICER), a powerful repressor of cAMP induced responses and gene transcription (Lamas *et al.*, 1996, Walker *et al.*, 1998), was not detected. These results indicate that responses to cAMP induction in the target cell by CyaA are important and together with the previous data represent an important role for the activation of cAMP induced transcription factors in the CyaA-induced immune response. An interesting further study would be to compare A-CyaA and iAC-CyaA stimulated macrophages by microarray to determine the role of cAMP induction in the modulation of innate immune responses at a transcriptional level.

A study by the Relman laboratory (Belcher *et al.*, 2000) analysed the responses of epithelial cells to *B. pertussis* by microarray. These results demonstrated the

activation of DNA binding proteins and immune response genes. Consistent with the results presented here, an increase in the transcription of A20, IL-1β, NFKBIA and ICAM-1 and also a down regulation of TNF receptors and cyclins involved in the cell cycle was observed. This indicates that CyaA may play an important role in the activation of immune responses to *B. pertussis*. The study by Belcher *et al.* also compared transcriptional responses of cells to wild type bacteria and those carrying a mutant PT gene. Their results indicated that down regulation of matrix-metaloproteinases (MMP) was an important PT induced step in responses to *B. pertussis*. A similar modulation of MMP genes was not observed here in macrophages in response to CyaA. An interesting further study would be to compare the transcriptional responses of innate immune cells to wild type *B. pertussis* and a strain deficient in CyaA. This would help to further elucidate the role of CyaA-induced responses during infection.

These results underline the complexity of the activation of immune responses induced by CyaA. The results here demonstrate that the regulatory/anti-inflammatory immune response elicited by CyaA and described in the previous chapters is polarised through the modulation of both pro- and anti-inflammatory response genes. To better understand this, the results are discussed further below under different heading.

7.3.1 Activation of regulatory and anti-inflammatory response genes by CyaA

In this study it was found that CyaA stimulation of macrophages resulted in the activation of genes associated with regulatory and anti-inflammatory immune functions. CD69 (also called very early activation antigen (VEAA)) was upregulated 0.5 h after co-stimulation of macrophages with CyaA and CpG and after 6 h by treatment with CyaA alone. CD69 plays an important role in preventing the autoimmune disease collagen induced arthritis (CIA) (Sancho *et al.*, 2003). Engagement of CD69 protects against CIA, by inducing TGF-β production, which in turn, suppresses the production of the inflammatory cytokine, IL-1β. Engagement of CD69 and subsequent production of TGF-β is also important in preventing the clearance of tumours (Esplugues *et al.*, 2003). These data demonstrate an important role

for CD69 in the generation of a regulatory cytokine environment. Induction of CD69 by CyaA may indicate a mechanism for TGB-β production, which is an anti-inflammatory cytokine and plays a role in promoting the induction of regulatory T cells.

Fcgr2b gene expression was upregulated by co-treatment of macrophages with CyaA and CpG. Fcgr2b (Fc receptor, IgG, low affinity IIb; also known as FcγRIIB) is an inhibitory receptor for IgG expressed primarily on myeloid cells, including DC. FcγRIIB stimulation plays a role in inhibiting antigen presentation by innate immune cells and leads to the induction of regulatory T cell (Samsom *et al.*, 2005). Upregulation of Fcgr2b gene expression by CyaA may represent a pathway by which the host induces regulatory type immune responses to limit immune mediated pathology during *B. pertussis* infection.

ICOSL mRNA expression was induced in macrophages following treatment with CyaA for 6 h. ICOSL has been shown to be essential to control the inflammatory response in asthma, through the induction of antigen-specific regulatory T cells (Akbari *et al.*, 2002). The upregulation of ICOSL by CyaA may represent a mechanism whereby *B. pertussis* induces T regulatory cells during infection, which help to prevent clearance of the bacteria by the immune system. Alternatively, the induction of Tr1 cells may be a host response to prevent excessive immune mediated damage during clearance of the bacterium.

TLR2 gene expression was upregulated 0.5 h after co-treatment with CyaA and CpG. TLR2 is the PRR expressed by innate cells necessary for the recognition of peptidoglycan from Gram-positive bacteria and yeast and is important in the resolution of these infections (Yoshimura *et al.*, 1999). While the development of TLR2-mediated immune response against infection is dependant on MyD88 (Power *et al.*, 2004), subsequent upregulation of TLR2 expression is MyD88 independent (Nilsen *et al.*, 2004). TLR2 activation has been shown to inhibit B cell development (Hayashi *et al.*, 2005). Recently, TLR2 dependant induction of *Candida*-specific Tr1 cells and the suppression of innate immunity has been reported (Netea *et al.*, 2004). TLR2 stimulation also promotes the secretion of high levels of IL-10 from DC (Dillon *et al.*, 2004). The upregulation of TLR2 expression by CyaA may represent a mechanism that contributes to the induction of IL-10-secretion and the generation of Tr1 cells.

CD47 gene expression was upregulated 6 h after co-treatment of macrophages with CyaA and CpG. CD47 expression promotes apoptosis and is also a positive regulator of phagocytosis (Manna *et al.*, 2005, Tada *et al.*, 2003). However, CD47 is part of the surface receptor to which FHA of *B. pertussis* binds on innate immune cells. FHA is an important adhesin molecule of *B. pertussis* and is necessary for successful infection. The enhancement of CD47 on target cells by CyaA may represent a mechanism whereby *B. pertussis* enhances its ability to interact with innate immune cells. In addition, FHA, like CyaA, is also a potent modulator of immune responses, inducing IL-10 secretion from innate cells and promoting the development of IL-10-secreting Tr1 cells *in vivo*. By promoting CD47 and its subsequent interaction with FHA, CyaA may promote the development of this regulatory immune phenotype, which is important in limiting immune mediated pathology during infection.

Fos (c-fos; FBJ osteosarcoma oncogene) is a transcription factor and is upregulated 0.5 h after treatment with CyaA alone and also 0.5 h after co-treatment with CyaA and CpG. Increased c-fos expression also promotes the development of B cells but also promotes the induction of apoptosis (Hu *et al.*, 1996, Yamashita *et al.*, 2005). c-fos is also necessary for optimal of TLR2-agonist induced production IL-10 (Dillon *et al.*, 2004), and is also required for suppression of IL-12p70 secretion. In addition, c-fos promotes the development of Th2 cells (Agrawal *et al.*, 2003). The upregulation of c-fos expression by CyaA may represent a mechanism through which CyaA can promote the induction of IL-10 from innate cells, which is critical for the induction of Th2 and Tr1 cells. In addition, CyaA can subvert the suppression of c-fos by CpG and upregulate expression when cells are co-treated with CyaA and CpG.

CCL4 (also known as MIP-1β) was upregulated 0.5 h after co-stimulation of macrophages with CyaA and CpG. MIP-1β has been shown to promote the induction of antigen specific IgG1 and IgG2a antibodies and plays a role in the activation of antigen-specific T cells, promoting both IFN-γ producing Th1 cells and also IL-4, IL-5 and IL-10 producing Th2 cells (Lillard *et al.*, 2003). This induction of MIP-1β gene expression may play a role in the activation of Th2 cells *in vivo* by CyaA as demonstrated in chapters 4, 5 and 6.

CCR5, the receptor for MIP-1β was also upregulated in macrophages after 6 h treatment with CyaA, which would promote the binding of MIP-1β and also facilitate migration of innate cells. Increased expression of CCR5 has been shown to reduce graft versus host disease (GVH) (Welniak *et al.*, 2004) and to promote tumor growth (Ng-Cashin *et al.*, 2003), both of which indicate the induction of an anti-inflammatory or regulatory immune environment.

IL1rn (also known as IL-1 receptor antagonist (IL-1Ra)) gene expression is upregulated by co-treatment of macrophages with CyaA and CpG for 0.5 and also treatment with CyaA alone for 6 h. IL-1Ra binds to IL-1R and blocks the activity of IL-1. IL-1Ra plays a role in preventing autoimmune diseases, including RA, through the inhibition of IL-1β-induced TNF-α and CD40 expression (Horai *et al.*, 2004). It is possible, that the suppression of TNF-α secretion and reduction in CD40 expression by CyaA, demonstrated in chapters 4 and 5, may be mediated in some part by the upregulation of IL-1Ra expression. Stimulation of macrophages with the TLR4-ligand LPS in the presence of IL-10 has been shown to upregulate IL-1Ra production (Carl *et al.*, 2004). However, IL-10 stimulation alone was not sufficient to increase IL-1Ra expression. IL-10 produced by innate cells in response to CyaA co-stimulation with a TLR-ligand, demonstrated in chapters 4 and 5, may be the mechanism by which CyaA promotes the production of IL-1Ra thereby reducing the inflammatory immune response.

IL-1r2 (also known as IL-1 receptor type II (IL-1Rβ)) is upregulated by co-incubation of macrophages with CyaA and CpG for 0.5 h and by CyaA alone at 6 h. IL-1Rβ is a decoy receptor for IL-1 and is usually membrane bound, but can be secreted as a soluble factor (Bourke *et al.*, 2003). IL-1Rβ expression is increased during systemic infections and severe inflammatory responses and appears to be a mechanism by which the host regulates the inflammatory response preventing severe immune mediated pathology. Treatment with anti-inflammatory agents, such as glucocorticoids, enhance expression of IL-1Rβ and indicates a role for inhibition of IL-1 responses in anti-inflammatory treatments (Muller *et al.*, 2002). The increased expression of IL-1Rβ, and indeed IL-1Ra, by CyaA may represent a mechanism whereby CyaA mediates its anti-inflammatory effects.

SOCS3 gene expression was induced 0.5 h after by treatment of macrophages with CyaA and CpG for 0.5 h, and also by CyaA alone after 6 h treatment. SOCS3 is an important signalling molecule involved regulating signalling in innate immune cell responses to pathogens and is also preferentially expressed in Th2 cells (Egwuagu *et al.*, 2002). SOCS3 is induced by IL-10 and inhibits LPS induced activation of innate cells (Berlato *et al.*, 2002). Infection with HSV or *Leismania* promotes SOCS3 expression promoting their survival in the host (Bertholet *et al.*, 2003, Yokota *et al.*, 2004). It is possible, therefore, that CyaA promotes the activation of SOCS3 through IL-10 produced in response to co-treatment with TLR ligands.

Nfkbia (also known as IκBα) together with IκBβ and IκBε form the IκB complex that sequesters NF-κB in the cytosol and prevents its translocation across the nuclear membrane into the nucleus to activate gene transcription. IκBα gene expression was upregulated in macrophages 0.5 h after co-treatment with CyaA and CpG and by CyaA alone at 6 h. Degradation of IκBα occurs after pro-inflammatory stimulation of cells and results in the activation of early inflammatory responses, including TNF-α, IL-1 and ICAM-I expression (Fan *et al.*, 2004). IκBα can be induced by NF-κB by a feedback loop thereby preventing a damaging inflammatory responses. The induction of IκBα gene expression by CyaA may represent a mechanism for activation of an anti-inflammatory pathway and may explain how CyaA suppresses TNF-α secretion and ICAM-I expression (demonstrated in chapters 4 and 5).

Nfkb1 gene expression was upregulated in macrophages following 6 h treatment with CyaA alone. The Nfkb1 gene product is a 105 kDa protein that acts as a transcriptional inhibitor and promotes the development of a Th2 type adaptive immune response (Artis *et al.*, 2002). Activation of Nfkb1 gene expression may represent a mechanism whereby CyaA promotes the generation of Th2 cells.

JunB is a transcription factor involved in the differentiation of immune cells. Stimulation of macrophages with CpG in the presence of CyaA resultd in the upregulation of *JunB* gene transcription at 0.5 hr following treatment. JunB promotes the differentiation of IL-4 secreting Th2 cells, and this preferential induction of IL-4 can also induce IL-13 and IL-5 and the regulatory cytokine IL-10 (Gao *et al.*, 2004,

Voice *et al.*, 2004). JunB may therefore, play a role in the generation of antigen-specific Th2 and Tr1 cells by CyaA *in vivo*.

Bcl3 was upregulated 0.5 h in macrophages after co-stimulation with CyaA and CpG for 0.5 h. Bcl3 is induced by IL-10 and its activation inhibits LPS induced TNF-α, but not IL-6 (Kuwata *et al.*, 2003). Bcl3 may become activated by the induction of IL-10 by CyaA and could play a role in the mechanism of inhibition of LPS and CpG induced TNF-α by CyaA.

Usp18 (also known as Usp15) encodes ubiquitin specific protease 18 and its expression was upregulated 0.5 h after treatment of macrophages with CyaA and CyaA for 0.5 h. Usp18 is necessary for viral RNA replication in cells infected with LCMV. However, Usp18 is a negatives regulator of type-1 IFN signalling (Ritchie *et al.*, 2004). The activation of Usp18 by CyaA sugests an alternative mechanism whereby CyaA may regulate the immune response.

Tnfaip3 (also known as A20) is a zinc finger transcription factor and is upregulated in macrophages after 0.5 h treatment with CyaA and CpG and also after 6 h treatment with CyaA alone. A20 protects against inflammation by interrupting TLR signalling by inhibiting TRAF6 and thereby preventing NF-κB activation (Boone *et al.*, 2004). A20 deficient mice are resistant to endotoxin induced shock. Activation of A20 has also been shown to protect mice from inflammation and promotes proliferation in the liver following damage or surgery by inhibiting NF-κB (Longo *et al.*, 2005). It is possible that CyaA induced expression of A20 inhibits pro-inflammatory signalling by TLR ligands that normally result in IL-12p70 and TNF-α production and CD40 and ICAM-I expression. A20 activation by CyaA may therefore play an important role in immunomodulation by CyaA.

The activation of genes that suppress inflammation, including IL-1Ra, IL-1Rβ, IκBα, BCL3 and A20, and genes that promote IL-10 secretion and the generation of a regulatory immune response, such as Fcgr2b, CD47, TLR2, SOCS3 and c-fos, by CyaA may indicate a mechanism through which *B. pertussis* has evolved to subvert the host immune response. By inhibiting inflammation CyaA prevents the elimination of *B. pertussis*. Howeveer, it is also possible that the host has evolved to recognise CyaA as a danger signal, and generates regulatory cells and Th2 cells through the expression of

genes such as Fcgr2b, TLR2, c-fos, MIP-1β, Nfkb1 and BCL3, to prevent immune mediated pathology.

7.3.2 Activation of pro-inflammatory response genes by CyaA

The generation of a potent inflammatory response, characterised by the secretion of inflammatory cytokine, including IL-12p70, from innate immune cells and the secretion and the induction of Th1 cells is critical for the elimination of *B. pertussis* and the resolution of the infection. The host has evolved many mechanisms to recognise *B. pertussis* and to mount an immune response against disease. The recognition of pathogen-derived molecules and virulence factors by cells of the innate immune system is mediated by PRRs and results in the activation of many intracellular signalling processes that ultimately lead to the generation of a potent inflammatory immune response that clears the infection. CyaA is a potent immunomodulator that targets innate immune cells and is an essential virulence factor of *B. pertussis*.

IL-1β was upregulated in macrophages 0.5 h after co-treatment for with CyaA and CpG, and also after 6 h treatment with CyaA or CpG alone treatment. IL-1β is a powerful inflammatory cytokine and mediates the effects of endotoxic shock, LPS induced neurotoxicity and graft rejection and also plays a role in diabetes (Emanuelli *et al.*, 2004, Suzuki *et al.*, 2004). However, IL-1β also plays a role in the activation of IL-6 and promotes the growth and angiogenesis of tumours, which requires an immunosuppressive environment (Parker *et al.*, 2002, Voronov *et al.*, 2003). In addition, IL-1β activates SOC3. It is possible therefore that while CyaA induces the expression of IL-1β the pro-inflammatory roles of IL-1β are abrogated by the upregulation of IL-1Ra and IL-Rβ by CyaA, while leaving the anti-inflammatory activities unaffected.

IL-15 gene expression is induced by treatment of macrophages with CyaA alone for 6 h. IL-15 plays a role in the pathogenesis of many inflammatory autoimmune conditions, including arthritis and GVH disease and also promotes the activation of NK and CTL responses that are involved in the clearance of *M. tuberculosis* infections (Blaser *et al.*, 2005, McInnes *et al.*, 2003, Umemura *et al.*, 2001). This indicates that IL-15 may be induced by the host in response to CyaA to aid clearance of a *B. pertussis* infection.

EBi3 (Epstein Barr virus induced gene 3) gene expression is upregulated by co-treatment with CyaA and CpG. EBi3 together with the p28 subunit, forms the cytokine

IL-27. IL-27 synergises with IL-12 to promote differentiation and proliferation of Th1 cells and the secretion of IFN-γ (Pflanz *et al.*, 2002). Increased expression of EBi3 may represent a mechanism through which the host increases production of IL-27 and promotes the generation of Th1 cells required to clear *B. pertussis* infection.

CXCL2 (MIP-2) also plays a role in promoting inflammatory responses against bacteria (Skidgel *et al.*, 2002). The upregulation of MIP-2 by innate cells in response to CyaA treatment may represent a host response designed to aid clearance of *B. pertussis* infection.

TLR3 gene expression was upregulated after 6 h treatment of macrophages with CyaA alone. TLR3 is a PRR for double stranded viral RNA and is also activated by the synthetic molecule poly I:C. Activation of TLR3 is important in the recognition of viruses and the production of type-1 interferons and also results in the secretion of CCL2 (MCP-1), MIP-1α, MIP-2 and IL-6 (Patole *et al.*, 2005). The induction of TLR3 expression by CyaA does seem somewhat counter-intuitive given that CyaA is derived from a Gram-negative bacterium that does not normally produce double stranded RNA. However, by upregulating TLR3 expression, CyaA increase the ability of the macrophage to respond to any viral challenge and in turn secrete IL-6, which is a characteristic cytokine upregulated by CyaA treatment with TLR ligands. Furthermore, the potential to produce MIP-2 (CXCL2) is consistent with the findings that CyaA enhances production of this chemokine. The potential for CyaA treated macrophages to produce MIP-1α is also consistent with the observation that CyaA treatment upregulates the expression of CCR5, which is a receptor for MIP-1α and also MIP-1β.

Expression of **CCR1 (MIP-1αR)** in macrophages was also upregulated after 6 hr treatment with CyaA. Although, it was observed that CyaA suppressed MIP-1α secretion from innate cells (chapter 5). This may indicate that CyaA enhances the ability of macrophages to migrate toward a chemokine stimulus but reduces their ability to recruit other immune cells to this location.

The product of **Nfkb1** gene expression, which is upregulated 6 h after treatment of macrophages with CyaA for 6 h, can be post-translational processed to a 50 kDa protein. This forms the DNA binding subunit of the NF-κB complex and is required for optimal Th1 development and bacterial clearance (Artis *et al.*, 2003). Upregulation of

Nfkb1 by CyaA may represent an important step in the activation of the host adaptive response against *B. pertussis* infection. Upregulation of Nfkb1 by CyaA seen following 6 h treatment with CyaA alone, may represent either a pro- or anti- inflammatory molecule depending on this post-translational processing. **IκbKε** (inhibitor of κB kinase ε) was upregulated 0.5 h after co-treatment of macrophages with CyaA and CpG or after 6 h treatment with CyaA. IκbKε is also upregulated in response to the inflammatory CpG stimulus after 6 h. IκbKε is a subunit of NF-κB and while NF-κB activation proceeds normally in IκbKε deficient cells, an additional family of leucine zipper-type transcription factors are not activated in response to LPS stimulation (Kravchenko *et al.*, 2003). These transcription factors play a role in the inflammatory response and their activation may be promoted by the increased expression of IκbKε by CyaA, which may reflect a mechanism for the initiation of an inflammatory response to *B. pertussis* infection and can be induced by TLR8 signalling.

MyD88 gene expression was upregulated in macrophages 6 h following treatment with CyaA alone. MyD88 is the adapter molecule used by TLRs the IL-1 receptor family to induce optimal immune responses against pathogens. MyD88 is required for the induction of inflammatory responses, including IL-12 production and also for the induction of Th1 cells to protect again pathogens (Muraille *et al.*, 2003). **T2b2** (TRAF2 binding protein) is also upregulated 6 h after co-treatment of macrophages with CyaA and CpG. T2b2 mediates the interaction between TRAF1 and TRAF6 and is important in intracellular signal transduction following IL-1 and TLR signalling (Takatsuna *et al.*, 2003). In addition, CyaA upregulates **TRAF1** expression after 6 h treatment. The upregulation of MyD88 and T2b2 by CyaA indicates that CyaA is increasing the potential of innate cells to respond to TLR stimuli and may facilitate CyaA synergy with other TLR-ligands.

ICOSL gene expression is upregulated 6 h after treatment of macrophages with CyaA. ICOSL is important in the activation of T helper cells and the promotion of humoral immunity against pathogens (Wong *et al.*, 2003). **ICAM-I** gene expression was enhances 0.5 h after CyaA and CpG co-stimulation of macrophages. However, a reduction in ICAM-I surface expression was observed on DC by FACS following treatment with CyaA; CyaA also suppressed CpG induced upregulation of ICAM-1

(chapters 4 and 5). TNF-α plays a role in the upregulation of ICAM-I expression (Qin *et al.*, 2005). However, the results presented in previous chapters demonstrate that CyaA downregulates TNF-α secretion by DC and macrophages. It is possible that the inhibition of CpG induced TNF-α by CyaA results in a post-transcriptional suppression of ICAM-I expression by innate cells.

Tnfrsf5 gene expression was also upregulated 6 h after CyaA treatment. Tnfrsf5 encodes CD40, and was also upregulated by CpG treatment for 6 h. Expression of CD40 and binding to CD40L (CD154) induces activation of DC and macrophages and the secretion of pro-inflammatory cytokines including IL-1, TNF-α, IL-12 and IL-15 (Haase *et al.*, 2004). CD40 is also required to activate T cells, and promote proliferation and IFN-γ production. However, expression of CD40 on the surface of DC was reduced following treatment with CyaA (chapter 4 and 5). CyaA also suppressed CpG induced expression of CD40. Blocking of CD40 activity induces a potent regulatory T cell population *in vivo* (Taylor *et al.*, 2002). It is possible that there is some mechanism whereby CyaA suppresses the translation of Tnfrsf5 mRNA and thereby suppresses CD40 expression by innate immune cells. This suppression of CD40 by CyaA would promote the generation of regulatory T cells by modulated innate immune cells.

The activation of expression of pro-inflammatory response genes by the host in response to CyaA treatment is an indication of how the host responds to *B. pertussis* infection. The expression of IL-27, IL-1β and MIP-2 by macrophages indicates that a potent inflammatory immune response is being mounted. In addition, treatment with CyaA appears to prime the innate cells for further activation with pathogen derived stimuli through the upregulation of TLR3 and CCR5. Furthermore, the activation of genes involved in the intracellular cascades that are activated by pathogen-derived danger signals indicates that the an inflammatory response is being promoted by treatment with CyaA. However, it appears that CyaA is also activating anti-inflammatory responses to counteract this. ICAM-I and CD40 gene expression is upregulated, but a reduction in surface expression of the molecules was observed. CCR1 is also upregulated while it's ligand, MIP-1α, is suppressed by CyaA. In addition, IL-1β gene expression was strongly upregulated, while IL-1Ra and IL-1Rβ were also upregulated. The induction of molecules that inhibit the host inflammatory

responses may represent a mechanism employed by *B. pertussis* to promote the generation of a regulatory immune response and thereby facilitate infection.

7.3.3 Activation of pro- and anti-apoptosis response genes by CyaA

In addition to immune modulatory functions, CyaA has been shown to lyse target cells and induce apoptosis in macrophages (Khelef *et al.*, 1994). Gene expression analysis revealed the upregulation of both pro- and anti-apoptotic factors by CyaA.

HSP105 is a heatshock protein that directly interacts with HSP70 heterodiners and inhibits their chaperonin activity (Yamagishi *et al.*, 2004). HSP105 is also a pro-apoptotic factor and increases oxidative stress induced apoptosis (Yamagishi *et al.*, 2002a, Yamagishi *et al.*, 2002b). HSP105 gene expression was induced 0.5 h after co-treatment of macrophages with CyaA and CpG and after 6 h treatment with CyaA alone.

Ier2 expression was upregulated 0.5 h following co-treatment with CyaA and CpG. Ier2 is a pro-apoptotic signalling factor and its activation has been shown to cause cell death in neuronal cells (Schneider *et al.*, 2004). Upregulation of HSP105 and Ier2 gene transcription by CyaA may reflect a mechanism where by cell function is inhibited and apoptosis is induced.

Crem expression was induced by co-treatment of macrophages with CyaA and CpG and also by treatment with CyaA alone at 6 h. Activation of Crem inhibits proliferation of cells and reduces the activity of macrophages (Sawka-Verhelle *et al.*, 2004).-**Btg2** (B cell translocation gene 2; also known as TIS21) is upregulated by CyaA after treatment for 0.5 h. Btg2 induces G2/M cell cycle arrest by inhibiting the transcription of cyclin D1 (Canzoniere *et al.*, 2004, Ryu *et al.*, 2004). **JunB** also has a negative effect on the progression of the cell cycle and through the suppression of cyclin D activation, which results in cell cycle arrest in the G1 phase (Andrecht *et al.*, 2002). Co-treatment of macrophages with CyaA and CpG upregulated JunB gene transcription following 0.5 h treatment, indicating that CyaA is inhibting cell cycle progression. In addition, Cyclin D1 (**Ccnd1**) is suppressed by co-treatment with CyaA and CpG for 6 h. **Ccng2** (Cyclin G2) was induced in macrophages 0.5 h after treatment with CyaA. Preliminary reports indicate that it inhibits cell proliferation (Tian *et al.*, 2002). The induction of Crem, Btg2, JunB and Ccng2 expression by CyaA indicates a mechanism whereby CyaA induces cell cycle arrest thereby inhibiting the function of

innate cells. This may reveal a pathway through which *B. pertussis* regulates the innate immune response to avoid clearance of the infection.

However, anti-apoptotic factors were also induced by treatment of macrophages with CyaA. **CXCL2 (MIP-2)** was upregulated by co-stimulation of macrophages with CyaA and CpG. CXCL2 plays a role in promoting cell survival through the inhibited apoptosis through the activation of the MAP kinase pathway (Watson and Fan, 2005). **CCL4 (MIP-1β)** was upregulated 0.5 h after stimulation of macrophages with CyaA in the presence CpG and 6 h after CyaA alone treatment. **CCR5**, the receptor for MIP-1β was also upregulated 6 h after treatment of macrophages with CyaA. MIP-1β is produced by neutrophils exposed to hypoxic conditions and promotes survival and inhibits the induction of apoptosis (Walmsley *et al.*, 2005). In addition, **Hifla** gene expression has been shown to promote the survival of neutrophils exposed to hypoxic conditions (Walmsley *et al.*, 2005). Hifla expression was upregulated 6 h after co-treatment of macrophages with CyaA and CpG.

Nfkbia (IκBα) has also been shown to play a role in the inhibition of apoptotic (Brouard *et al.*, 2002) and is induced by co-treatment with CyaA and CpG for 0.5 h. While **IL-15** has many pro-inflammatory properties, it has also been shown to inhibit apoptosis induced by TNF-α and endotoxic shock (Hiromatsu *et al.*, 2003) and was induced 6 h after treatment with CyaA. Activation of MIP-2, MIP-1β, CCR5, Hifl, Nfkbia and IL-15 by the host in response to CyaA may represent a series of host responses that counteract *B. pertussis* induced apoptosis. This activation of anti-apoptotic gene expression may promote the survival of innate cells and the clearance of *B. pertussis* infection.

7.3.4 Modulation of anti-bacterial response genes by CyaA

Recruitment of inflammatory cells into the lungs of infected mice is essential for the clearance of *B. pertussis* infection and abolition of CyaA expression in *B. pertussis* disrupts this cellular recruitment (Gross *et al.*, 1992, Harvill *et al.*, 1999, Khelef *et al.*, 1994, Weiss *et al.*, 1984). Co-treatment of macrophages with CyaA and CpG induced the expression of **CXCL2** (MIP-2), which has chemoattractant properties and promotes the recruitment of neutrophils and other immune cells. In addition, **IL-1β,** which is also

induced by co-treatment with CyaA and CpG, promotes neutrophil activation and survival (Prince *et al.*, 2004). However, CyaA has been shown to inhibit the functions of neutrophils, which include chemotaxis, phagocytosis and superoxide generation (Friedman *et al.*, 1987, Khelef *et al.*, 1994, Narayanan *et al.*, 2002, Pearson *et al.*, 1987). The release of chemoatractant molecules in response to CyaA treatment may represent a mechanism whereby *B. pertussis* can recruit immune cells to the site of infection to inhibit their function. Indeed, **Sod2** (Superoxide demutase 2) gene expression was upregulated by CyaA. Sod2 prevents superoxide accumulation (Friedman *et al.*, 2004), perhaps indicating a potential pathway for CyaA inhibition of neutrophil function at the site of *B. pertussis* infection.

7.3.5 Downregulation of pro-inflammatory genes by CyaA

CyaA synergises with TLR ligands to induce IL-10 secretion from innate immune cells and also promotes the generation of IL-10 secreting Tr1 cells *in vivo*. In addition, CyaA down regulates the expression of surface markers that promote pro-inflammatory immune responses. How CyaA exerts these anti-inflammatory effects at a transcriptionally level was not fully understood. Microarray analysis of macrophages revealed CyaA treatment suppressed the expression of genes encoding pro-inflammatory mediators and signalling molecules.

TLR4 gene expression is suppressed 0.5 h after treatment of macrophages with CyaA and CpG. TLR4 is the PRR that recognises Gram-negative bacterial LPS. TLR4 is required for development of immune responses against bacterial infections (Branger *et al.*, 2004, Power *et al.*, 2004). Signalling via TLR4 requires the recruitment of the adapter molecule MyD88 and the phosphorylation of IRAK1, IRAK4 and TRAF6 and the translocation of NF-κB to the nucleus (Akira, 2003, Iwasaki and Medzhitov, 2004). Direct downregulation of TLR4 by CyaA may suppress macrophage responses to bacteria. In addition, down regulation of TLR 4 may augment the suppression of TRAF6 activity by A20 (Boone *et al.*, 2004); A20 is upregulated in macrophages after treatment with CyaA. The downregulation of TLR4 gene transcription by CyaA may

play a role in the mechanism by which *B. pertussis* evades recognition by the immune system and suppresses inflammatory responses.

IL-7r (IL-7 receptor) gene expression is down regulated 0.5 h after treatment with CyaA. IL-7 is involved in the regulation of immune cell development and has been shown to exacerbate colitis, however blocking IL-7r inhibited inflammation and ameliorated colitis. (Okada *et al.*, 2005). The inhibition of IL-7r by CyaA may indicate that inflammatory responses mediated by IL-7 are suppressed and may facilitate the generation of anti-inflammatory and regulatory cells.

CCL12 gene expression is down regulated 0.5 h after treatment with CyaA. While no exact function for CCL12 has yet been identified, it belongs to a family of small secreted proteins with chemo-attractant properties, and therefore likely has similar functions (Kawai *et al.*, 2001a). Downregulation of CCL12 by CyaA may indicate a role for CCL12 in inhibiting the migration of inflammatory cells to the site of *B. pertussis* infection and may play a role in the suppression of anti-bacterial inflammatory responses.

Traf1 gene expression is down regulated 0.5 h after CyaA treatment. TRAF1 is an important intracellular signalling molecule that is required for optimal TLR-ligand and IL-1 stimulated inflammatory immune responses (Takatsuna *et al.*, 2003) In contrast, Traf1 gene expression is upregulated 6 h after CyaA treatment. These results may indicate that CyaA suppresses TLR and IL-1 signal transduction at early time points, but may enhance these signals at later timepoints. Early suppression of TRAF1 by CyaA may reveal a mechanism whereby regulatory immune responses can be established during the absence of an inflammatory immune environment. Interestingly, TRAF1 has recently been shown to inhibit MyD88 and TRIF mediated signalling (Su *et al.*, 2005) which may indicate a mechanism whereby CyaA could inhibit some aspects of TLR signaling.

Nfatc1 (NFAT2) gene expression is downregulated after 0.5 h co-treatment with CyaA and CpG and was also suppressed 6 h after treatment. NFAT2 is a transcription factor that promotes cytokine secretion in a variety of cell types (Monticelli and Rao, 2002). Expression of NFAT2 is dependent on TRAF6 (Takayanagi *et al.*, 2002). The suppression of NFAT2 by CyaA may be direct or may

be mediated though CyaA-induced activation of A20, which directly inhibits TRAF6 activity (Boone *et al.*, 2004). Normal development of B cells and subsequent humoral immune responses require NFAT2 expression (Berland and Wortis, 2003). In addition, NFAT2 expression is required for IL-4 secretion and the development of effector Th1 and Th2 cells (Brogdon *et al.*, 2002). Inhibition of NFAT2 by CyaA may play a role in promoting anti-inflammatory innate immune cell responses during *B. pertussis* infection.

Ccne2 (Cyclin E2) expression was down regulated 0.5 h after treatment of macrophages with CyaA and CpG and also downregulated after 6 h treatment. Cyclin E2 is a cytoplasmic protein that is required for the progression of the cell cycle is critical for proliferation of immune cells (Geng *et al.*, 2003). Suppression of Cyclin E2 by CyaA and also Cyclin D1 **(Ccnd1),** which is down regulated 6 h after co-treatment with CyaA and CpG, together with the upregulation of JunB and Btg2 that downregulates expression of Cyclin D, may indicate a mechanism through which cell cycle progression is halted and innate immune cell proliferation is inhibited.

During *B. pertussis* infection inhibition of innate immune cell responses by CyaA may play a role in the inhibition of the immune response during infection. The downregulation of receptors for external stimuli, such as TLR4 and IL-7r, and suppressing the expression of signalling molecules required to promote a pro-inflammatory immune response, such as TRAF1, NFAT2 and Cyclin E2, indicates that CyaA may inhibit pro-inflammatory responses required to eliminate infection.

7.3.6 Negative regulation of CyaA-induced anti-inflammatory responses by host cells

CyaA suppresses the expression of genes required for a potent inflammatory immune response. However, the host innate immune cell negatively regulates the expression of genes whose products suppress the inflammatory response.

Tgfbr2 (TGF-β receptor II) gene expression was down regulated 6 h after co-treatment with CyaA and CpG. Tgfbr2 is a subunit of the receptor for the anti-

inflammatory cytokine TGF-β. Disruption of the Tgfbr2 gene was shown to result in a lethal inflammatory disorder (Leveen *et al.*, 2002). Tgfbr2 mediates the induction of anti-proliferative signals and the inhibition of TLR-induced signalling (Roes *et al.*, 2003). Downregulation of TGF-β responsiveness in host innate immune cells in response to CyaA may indicate that the host down-regulates the anti-inflammatory responses induced by CyaA and *B. pertussis* during infection and may enhance the clearance of *B. pertussis.*

SMAD1 gene expression is down regulated by 0.5 h after co-treatment with CyaA and CpG. SMAD1 belongs to a family of intracellular signalling molecules that play a role in TGF-β signalling. TGF-β activates SMAD1 during tumor development and the inhibition of SMAD1 inhibits tumor cell proliferation. (Munoz *et al.*, 2004), indicating a role for SMAD1 activation in maintaining immuno-supressive regulatory immune environments. SMAD1 downregulation by the host innate cells in response CyaA may indicate that anti-inflammatory responses induced by *B. pertussis* that may facilitate the development of an inflammatory response required to clear infection.

CCL2 (MCP-1) gene expression is downregulated 0.5 h after treatment with CyaA. MCP-1 is powerful a chemo-attractant molecule for macrophages and T cells. Over-expression of MCP-1 in the CNS was shown to result in the amelioration of inflammation and EAE (Elhofy *et al.*, 2005). MCP-1 is also important for the suppression of IL-12 and IFN-γ responses during infection (deSchoolmeester *et al.*, 2003) and was shown to play the maintenance of oral tolerance (DePaolo *et al.*, 2003). TLR3-agonists promote the induction of MCP-1. Although MCP-1 expression is downregulated by CyaA the expression of TLR3 is upregulated. This may indicate that a negative regulator of TLR3-induced MCP-1 transcription may also be upregulated by CyaA. Downregulation of MCP-1 expression by the host in response to CyaA treatment may indicate that the host negatively regulates the supression of inflammatory responses by *B. pertussis*,

The induction of Tr1 cells and the secretion of the IL-10 and TGF-β by the host during infection prevents immune-mediated pathology. However, the induction of regulatory responses by pathogen-derived molecules may subvert the host responses and prolong the infection. The data here indicates that host responses may be activated

in response to CyaA that negatively regulate the induction pathogen-induced regulatory responses.

7.3.7 Modulation of gene Expression by CpG

CpG is a potent pro-inflammatory molecule that binds to TLR9 and promotes the inflammatory responses. Activation of innate immune cells by CpG induces the upregulation of the pro-inflammatory co-stimulatory molecules CD40 and ICAM-I and the secretion of the pro-inflammatory cytokines IL-12p70 and TNF-α. Microarray technology was used to analyses the modulation of gene expression in macrophages by CpG. It was found that only 8 genes were modulated by CpG 0.5 h after treatment. TLR9 primarily localises to intracellular compartments in innate cells (Takeshita *et al.*, 2001) and this may delay the activation of gene expression by CpG. In addition, CpG modulated 238 genes following 6 h after treatment of innate cells, which included genes associated with pro-inflammatory immune responces.

7.3.7.1 Upregulation of pro-inflammatory genes by CpG

CCL12 was upregulated following 6 h after treatment with CpG. No definite function has yet been attributed to CCL12, however CCL12 is structurally similar to the family of chemokines. CCL12 is therefore thought to be involved immune cell migration (Kawai *et al.*, 2001a). Upregulation of CCL12 by the host cell in response to CpG treatment may play a role in the activation of immune responses.

CCL9 (MIP-1γ) expression was upregulated in host cells 6 h after treatment with CpG. CCL9 plays an important role in the recruitment of DC (Zhao *et al.*, 2003). **CCL4 (MIP-1β)** expression was also upregulated 6 h following treatment with CpG. CCL4 is also a chemokine but is important for the development of IFN-γ secreting Th1 cells (Lillard *et al.*, 2003). Upregulation of CCL12, CCL9 and CCL4 in response to CpG may promote the recruitment of innate cells to sites of inflammation.

CXCL2 (MIP-2) expression was upregulated 6 h after treatment with CpG. MIP-2 promotes inflammatory responses against bacteria (Skidgel *et al.*, 2002). In addition, after 6 h treatment of macrophages with CpG **IL-1β** gene expression was

upregulated. IL-1β is a potent pro-inflammatory cytokine that mediates the effects of endotoxic shock, immune responses to bacteria and GVH disease (Suzuki *et al.*, 2004). The upregulation of MIP-2 and IL-1β by innate cells in response to CpG may indicate the activation of host responses designed to promote inflammation and to facilitate clearance of bacterial infections.

CD40 (**Tnfrsf5**) and **ICAM-I** gene expression were upregulated 6 h after treatment with CpG. Upregulation of CD40 and ICAM-I by innate cells promotes the generation of IFN-γ secreting Th1 cells (Moser and Murphy, 2000). In addition to the secretion of pro-inflammatory cytokines and chemokines, the upregulation of these co-stimulatory molecules by innate cells in response to CpG facilitates the induction of IFN-γ-secreting T h1 cells.

Jak2 (Janus kinase 2) gene expression was increased in cells 6 h following treatment with CpG. Jak2 is a signalling molecule that is phosphorylated immediately after LPS stimulation of TLR4 and is important for the production of IL-1β in response to TLR4-agonists (Okugawa *et al.*, 2003). In addition, Jak2 has been shown to mediate the development of Th1 cells by promoting IL-12 production from innate immune cells (Sugimoto *et al.*, 2003). Activation of Jak2 also promotes proliferation and inhibits apoptosis (Ruchatz *et al.*, 2003). The induction of Jak2 by CpG may indicate a mechanism by which signalling via TLR9 activates pro-inflammatory responses in innate cells, including IL1-β and IL-12 secretion.

TRAF1 gene expression was upregulated after 6 h treatment with CpG. TRAF1 is an important signalling molecule and is required for efficient transduction of TLR ligand and IL-1 stimuli and the induction of inflammatory immune responces (Takatsuna *et al.*, 2003). Upregulation of TRAF1 by CpG may indicate the enhancement of TLR signal transduction and activation of pro-inflammatory immune responses.

CpG motifs are present in bacterial DNA and induce potent pro-inflammatory responses. The activation of CCL12, CCL9, CCL4, CXCL2, IL-1β, CD40, ICAM-I, Jak2 and TRAF1 gene expression in macrophages in response to CpG reveals a complex cell signalling system that promotes pro-inflammatory immune responses.

7.3.7.2 Down regulation of anti-inflammatory genes in response to CpG

Innate immune cells recognise CpG as a danger signal from bacteria and upregulate pro-inflammatory response genes and anti-bacterial immune responses. The generation of anti-inflammatory responses during infection would inhibit the clearance of the pathogen. This study demonstrates that macrophages down regulate genes associated with anti-inflammatory responses following CpG stimulation.

c-fos gene expression was down regulated after 6 h treatment with CpG . c-fos expression promotes the development of Th2 cells and the secretion of IL-10 (Agrawal *et al.*, 2003, Dillon *et al.*, 2004). In addition, IL-12p70 secretion is increased in c-fos deficient mice (Dillon *et al.*, 2004). Suppression of c-fos expression by the host in response to CpG may indicate the activation of pathways that negatively regulate anti-inflammatory responses during bacterial infections.

Treatment of cells with CpG for 6 h downregulated the expression of the **Crem** gene. Crem expression has been shown to suppress cell proliferation and activation (Sawka-Verhelle *et al.*, 2004). Inhibition of Crem in response to CpG may promote the generation of immune responses. **Siva (CD27 binding protein)** is a positive regulator of apoptosis (Prasad *et al.*, 1997), which is down regulated in macrophages after 6 h treatment with CpG. Suppression of Siva in response to CpG may inhibit apoptosis of innate immune cells and facilitate the generation of inflammatory immune responses.

The suppression of c-fos, Crem and Siva expression in response to CpG may indicate a mechanism by which the host innate immune cells suppress the activation of anti-inflammatory responses during bacterial infection that would inhibit the generation of pro-inflammatory immune responses required to clear the pathogen.

7.3.7.3 Upregulation of anti-inflammatory genes in response to CpG

CpG is a potent pro-inflammatory molecule and activates the expression of genes associated with inflammation and bacterial clearance. In addition, the data here also demonstrates that CpG suppresses certain anti-inflammatory responses. However, evidence that CpG promotes the expression of anti-inflammatory genes was also found.

CpG treatment of macrophages induces the expression of ICOSL, CD69, Tnfaip3 and IL1rn gene expression. **ICOSL** is involved in the control of inflammation during asthma and the generation of antigen-specific regulatory cells (Akbari *et al.*, 2002). **CD69** expression is important in preventing arthritis and in promoting the induction of TGF-β secretion (Sancho *et al.*, 2003). **Tnfaip3 (A20)** expression inhibits pro-inflammatory signalling and protects against endotxin induced shock and inflammation (Longo *et al.*, 2005). **IL1rn** encodes IL-1Ra that is a receptor antagonist for IL-1R and inhibits the activity of IL-1β (Horai *et al.*, 2004). **IκBα** prevents the translocation of NF-κB across the nuclear membrane (Fan *et al.*, 2004). The upregulation of these genes may represent a mechanism through which regulatory cells are induced in during bacterial infection. The induction of regulatory T cells is thought to protect against immune mediated pathology. In addition, expression of **IL-7r** is suppressed by CpG. IL-7 mediates pro-inflammatory immune responses and suppression of IL-7r may indicate an addition method of employed by the host to regulate pro-inflammatory responses.

Pathogens inhibit the host immune responses by producing virulence factors that have direct effects on effector immune cells. However, it is becoming clear that regulatory cells are induced during infection with many pathogens. The induction of these cells by the host may regulate inflammation and immune mediated damage to host tissues. However, it is also possible that regulatory cells are induced by pathogens to subvert the host immune responses. CyaA is a potent immunomodulator that regulates innate cell function and promotes the induction Tr1 cells. In this study it was found that CyaA mediates the induction of Tr1 cells by modulating gene expression in innate cells. CyaA upregulates the expression of surface markers and secreted cytokines that promote the generation of regulatory cells. In addition, CyaA downregulates the expression of pro-inflammatory genes and upregulate the expression of molecules that are antagonist to inflammatory mediators. These results indicates that CyaA plays a key role in the modulation of the immune response during *B. pertussis* infection.

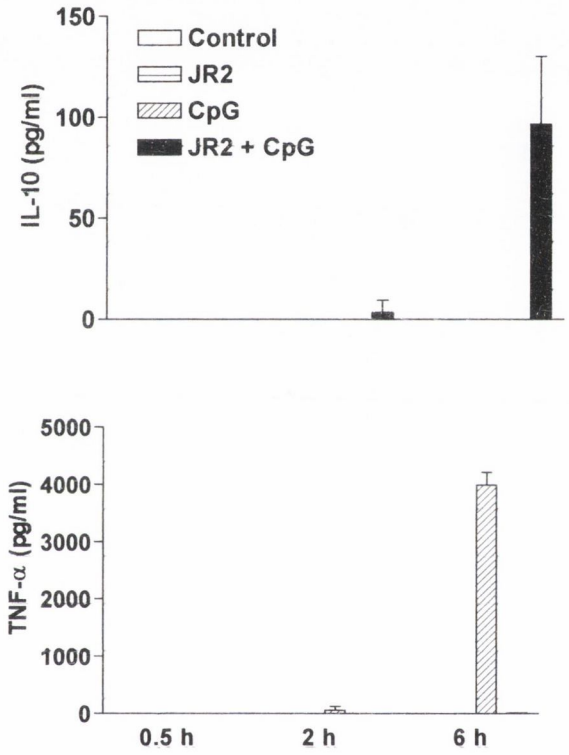

Figure 7.1 CyaA modulates TLR9 ligand induced cytokine secretion in macrophages.
J774 macrophages were treated with CyaA (1 μg) CpG (10 μg) or both, supernatants were collected after 0.5, 2 and 6 h and IL-10 and TNF-α concentrations tested by ELISA. CyaA synergises with CpG to promote IL-10 secretion from macrophages and suppresses CpG driven TNF- α production.

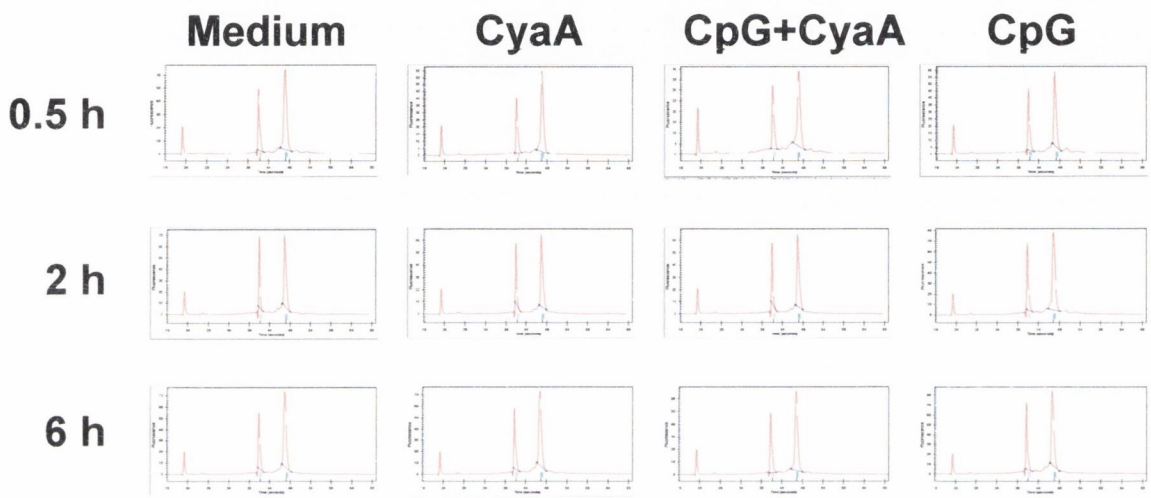

Figure 7.2 Bioanalyser traces of RNA purified from macrophages
J774 macrophages were treated with CyaA (1 μg) CpG (10 μg) or both. RNA was extracted 0.5 h, 2 h and 6 h later. Each sample was analysed using a Bioanalyser 2100 for quality and quantity. The 18S and 28S ribosomal RNA peaks were identified in each sample.

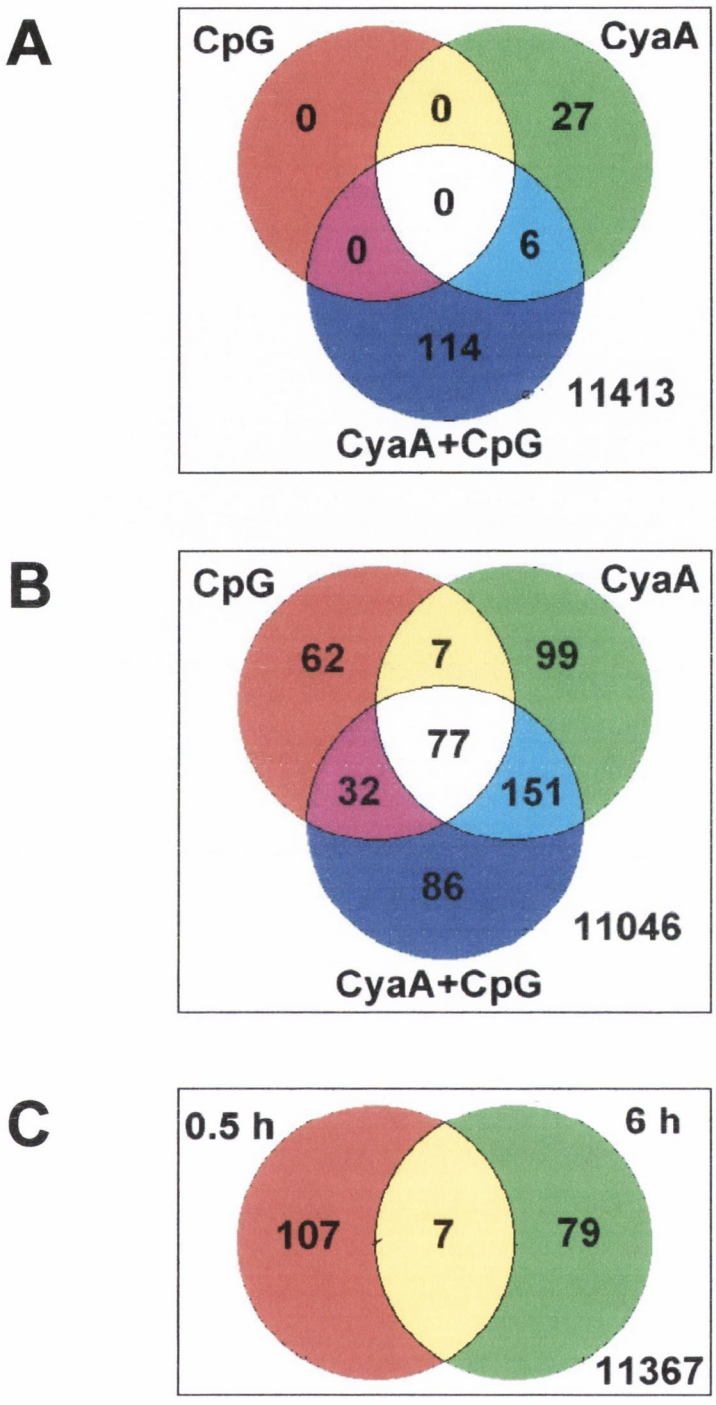

Figure 7.3 Upregulation of genes following treatment of macrophages with CyaA and CpG J774 macrophages were treated with CyaA (1 μg) CpG (10 μg) or both. RNA was extracted 0.5 h (A) and 6 h (B) later gene expression analyzed by GeneChip microarray. Genes upegulated by the three treatments were analysed and gene lists compared. Genes uniquely upregulated by CyaA plus CpG at 0.5 h and 6 h were compared (C).

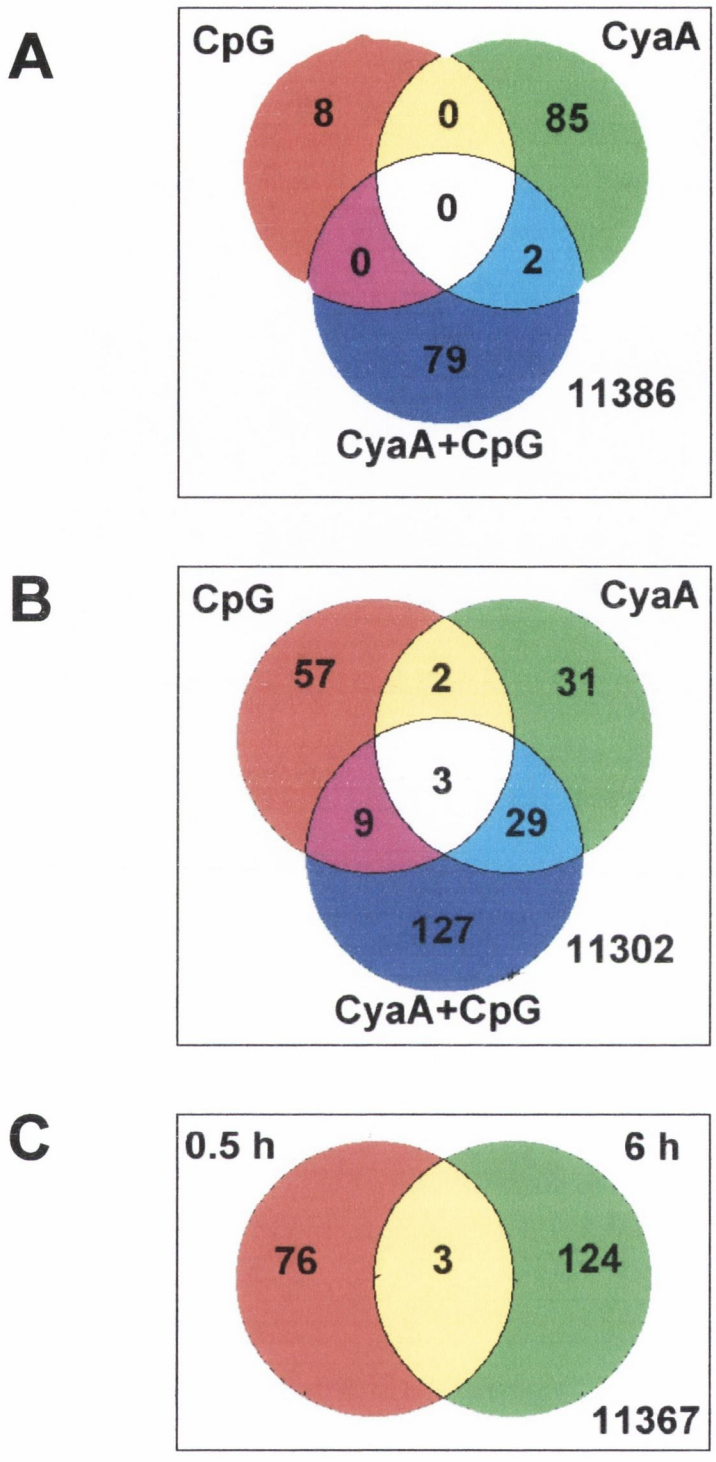

Figure 7.4 Downregulation of genes following treatment of macrophages with CyaA and CpG J774 macrophages were treated with CyaA (1 μg) CpG (10 μg) or both. RNA was extracted 0.5 h (A) and 6 h (B) later gene expression analyzed by GeneChip microarray. Genes downregulated by the three treatments were analysed and gene lists compared. Genes uniquely downregulated by CyaA plus CpG at 0.5 h and 6 h were compared (C).

Table 7.1 Genes upregulated by both CyaA and co-treatment with CyaA and CpG at 0.5 h			
Gene Name	Fold Change	Name	Description
1423100_at	7.91	Fos	FBJ osteosarcoma oncogene
1457404_at	4.886	AA408868	expreexpressed sequence AA408868
1448890_at	3.835	Klf2	Kruppel-like factor 2 (lung)
1417483_at	3.339	AA408868	expreexpressed sequence AA408868
1437868_at	3.318	BC023892	uw52c03.x1 Soares_mammary_gland_NMLMG cDNA clone mRNA sequence.
1448728_a_at	3.104	AA408868	expreexpressed sequence AA408868

Table 7.2 Genes uniquely upregulated by treatment with CyaA for 0.5 h			
Gene Name	Fold Change	Name	Description
1422612_at	4.995	Hk2	hexokinase 2
1416250_at	4.93	Btg2	B-cell translocation gene 2, anti-proliferative
1416488_at	4.564	Ccng2	cyclin G2
1448364_at	4.522	Ccng2	cyclin G2
1456909_at	4.296		Transcribed sequence with moderate similarity toPOL2
1428306_at	4.198	Ddit4	DNA-damage-inducible transcript 4
1442027_at	4.176	A530083I02Rik	RIKEN cDNA A530083I02 gene
1442710_at	4.102		Transcribed sequences
1441112_at	4.089		BB394889 RIKEN 0 day neonate cerebellum cDNA clone, mRNA
1427322_at	3.825	Wdr9	WD repeat domain 9
1455773_at	3.676		Transcribed sequence S12207 hypothetical protein
1438781_at	3.447		Adult male aorta and vein cDNA, RIKEN unknown EST
1441677_at	3.392		Transcribed sequence with similarity to NP_081764.1 RIKEN cDNA
1447706_at	3.38		BB535847 RIKEN full-length enriched mRNA sequence.
1460369_at	3.363	BC003267	similar to zinc finger protein 97
1452798_s_at	3.293	2310009E07Rik	RIKEN cDNA 2310009E07 gene
1441477_at	3.284	9530075H20Rik	calumenin
1460699_at	3.257	Rps27	ribosomal protein S27
1459871_x_at	3.235	9530046H09Rik	AV349132 RIKEN full-length enriched mRNA sequence.
1428873_a_at	3.175	4121402D02Rik	RIKEN cDNA 4121402D02 gene
1458406_at	3.169		3 days neonate thymus cDNA, RIKEN unknown EST, full insert seq
1458972_at	3.081	9330112F22Rik	Adult male diencephalon cDNA, RIKEN full-length unknown EST
1457508_at	3.048	C430003N24Rik	7 days embryo whole body cDNA, RIKEN unknown EST
1439678_at	3.045		Transcribed sequences
1455300_at	3.027	E130014J05Rik	RIKEN cDNA E130014J05 gene
1444456_at	3.022	9030425P06Rik	choline/ethanolaminephosphotransferase 1
1431028_a_at	3.015	Pank1	pantothenate kinase 1

Table 7.3 Genes uniquely upregulates by co-treatment with CyaA and CpG for 0.5 h			
Gene Name	Fold Change	Name	Description
1449399_a_at	141.3	Il1b	interleukin 1 beta
1417262_at	31.52	Ptgs2	prostaglandin-endoperoxide synthase 2
1427736_a_at	31.39	Ccrl2	chemokine (C-C motif) receptor-like 2
1417263_at	25.47	Ptgs2	prostaglandin-endoperoxide synthase 2
1455899_x_at	21.27	Socs3	suppressor of cytokine signaling 3
1456212_x_at	16.14	Socs3	BB831725 RIKEN full-length enriched, mRNA sequence.
1424339_at	14.27	Oasl1	2'-5' oligoadenylate synthetase-like 1
1422473_at	12.67	Pde4b	phosphodiesterase 4B, cAMP specific
1450750_a_at	12.18	Nr4a2	nuclear receptor subfamily 4, group A, member 2
1435906_x_at	11.99	Gbp2	guanylate nucleotide binding protein 2
1427381_at	11.58	Irg1	immunoresponsive gene 1
1418322_at	10.66	Crem	cAMP responsive element modulator
1450672_a_at	10.64	Trex1	three prime repair exonuclease 1
1422474_at	9.583	Pde4b	phosphodiesterase 4B, cAMP specific
1421031_a_at	9.116	2310016C08Rik	RIKEN cDNA 2310016C08 gene
1436196_at	8.086	C030046G05	cDNA, RIKENunknown EST, full insert sequence
1420710_at	7.855	Rel	reticuloendotheliosis oncogene
1437199_at	7.681		Similar to MAP-kinase phosphatase (cpg21) (LOC240672), mRNA
1439819_at	7.673	AU015263	Transcribed sequences
1418932_at	7.673	Nfil3	nuclear factor, interleukin 3, regulated
1428735_at	7.431	Cd69	CD69 antigen
1450350_a_at	6.984	Jundm2	Jun dimerization protein 2
1446834_at	6.786		Transcribed sequences
1455900_x_at	6.523	Tgm2	BB041811 RIKEN full-length enriched mRNA sequence.
1419004_s_at	6.42	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a
1437939_s_at	6.363		Transcribed sequences
1444531_at	6.237		UI-M-AI1-afs-b-02-0-UI.s1 mRNA sequence.
1447284_at	6.185	Trem1	triggering receptor expressed on myeloid cells 1
1416273_at	6.114	Tnfaip2	tumor necrosis factor, alpha-induced protein 2
1435458_at	6.007	Pim1	mp37c03.x1 MPLRB1 cDNA clone IMAGE:571396 mRNA sequence.
1431843_a_at	5.992	Nfkbie	nuclear factor of kappa light gene enhancer in B-cells inhibitor, eps
1449037_at	5.942	Crem	cAMP responsive element modulator
1437226_x_at	5.908	Mlp	MARCKS-like protein
1415922_s_at	5.816	Mlp	MARCKS-like protein

1433699_at	5.783	Tnfaip3	tumor necrosis factor, alpha-induced protein 3
1460220_a_at	5.44	Csf1	colony stimulating factor 1 (macrophage)
1434794_at	5.433	Rhof	ras homolog gene family, member f (in filopodia)
1437277_x_at	5.399	Tgm2	transglutaminase 2, C polypeptide
1420549_at	5.361	Gbp1	guanylate nucleotide binding protein 1
1458299_s_at	5.342	Nfkbie	BB820441 RIKEN mRNA sequence.
1448724_at	5.261	Cish	cytokine inducible SH2-containing protein
1425732_a_at	5.19	Mxi1	Max interacting protein 1
1438855_x_at	5.17	Tnfaip2	BB233088 RIKEN mRNA sequence.
1433428_x_at	5.076	Tgm2	transglutaminase 2, C polypeptide
1425412_at	5.03	Cias1	cold autoinflammatory syndrome 1 homolog (human)
1425993_a_at	4.986	Hsp105	heat shock protein 105
1452418_at	4.97	1200016E24Rik	mab33d12.x1 Soares_NMEBA mRNA sequence.
1423566_a_at	4.952	Hsp105	heat shock protein 105
1437247_at	4.69	Fosl2	fos-like antigen 2
1417372_a_at	4.523	Peli1	pellino 1
1419132_at	4.496	Tlr2	toll-like receptor 2
1435627_x_at	4.417	Mlp	Transcribed sequences
1449984_at	4.409	Cxcl2	chemokine (C-X-C motif) ligand 2
1424067_at	4.373	Icam1	intercellular adhesion molecule
1454976_at	4.342		Adult male urinary bladder cDNA unknown EST,
1450165_at	4.291	Slfn2	schlafen 2
1420664_s_at	4.256	Procr	protein C receptor, endothelial
1435454_a_at	4.248	BC006779	cDNA sequence BC006779
1451206_s_at	4.23	Pscdbp	pleckstrin homology, Sec7 and coiled-coil domains, binding protein
1452519_a_at	4.184	Zfp36	unnamed protein product; TIS11 (AA 1 - 183); mRNA.
1421578_at	4.148	Ccl4	chemokine (C-C motif) ligand 4
1418580_at	4.103	5830458K16Rik	RIKEN cDNA 5830458K16 gene
1422631_at	4.074	Ahr	aryl-hydrocarbon receptor
1419647_a_at	4.004	Ier3	immediate early response 3
1456661_at	3.976		Transcribed sequence with moderate similarity to protein pdb:1JZ5
1423274_at	3.967	Ddx26	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26
1420330_at	3.936	Clecsf9	C-type lectin, superfamily member 9
1425663_at	3.897	Il1rn	interleukin 1 receptor antagonist
1420331_at	3.864	Clecsf9	C-type lectin, superfamily member 9
1435697_a_at	3.831	Pscdbp	BB503614 RIKEN mRNA sequence.

1420425_at	3.827	Prdm1	PR domain containing 1, with ZNF domain
1438152_at	3.826	Gpaa1	BB550093 RIKEN mRNA sequence.
1435174_at	3.797	C230004D03Rik	RIKEN cDNA C230004D03 gene
1457445_at	3.766	Trps1	trichorhinophalangeal syndrome I (human)
1435415_x_at	3.763	Mlp	MARCKS-like protein
1448306_at	3.75	Nfkbia	nuclear factor of kappa light chain gene enhancer in B-cells inhibA
1417371_at	3.742	Peli1	pellino 1
1437884_at	3.725	Arl8	ADP-ribosylation factor-like 8
1423006_at	3.65	Pim1	proviral integration site 1
1427844_a_at	3.614	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
1435872_at	3.611	Pim1	proviral integration site 1
1420380_at	3.575	Ccl2	chemokine (C-C motif) ligand 2
1434881_s_at	3.554	Kctd12	potassium channel tetramerisation domain containing 12
1450376_at	3.511	Mxi1	Max interacting protein 1
1415972_at	3.428	Marcks	myristoylated alanine rich protein kinase C substrate
1428909_at	3.36	1200015M12Rik	C85657 mRNA sequence.
1449175_at	3.354	Gpr65	G-protein coupled receptor 65
1418946_at	3.351	Siat4a	sialyltransferase 4A (beta-galactoside alpha-2,3-sialytransferase)
1423520_at	3.349	Lmnb1	lamin B1
1442700_at	3.33	Pde4b	phosphodiesterase 4B, cAMP specific
1430752_at	3.329	C330006D17Rik	BB397948 RIKEN mRNA sequence.
1415899_at	3.291	Junb	Jun-B oncogene
1420342_at	3.288	Gdap10	pre-B-cell colony-enhancing factor 1
1419714_at	3.238	Pdcd1lg1	programmed cell death 1 ligand 1
1418133_at	3.227	Bcl3	B-cell leukemia/lymphoma 3
1418025_at	3.218	Bhlhb2	basic helix-loop-helix domain containing, class B2
1418183_a_at	3.216	Pscd1	pleckstrin homology, Sec7 and coiled-coil domains 1
1421408_at	3.201	Igsf6	immunoglobulin superfamily, member 6
1419068_at	3.2	Rabgef1	RAB guanine nucleotide exchange factor (GEF) 1
1436026_at	3.188	1110032O19Rik	18-day embryo whole body cDNA, RIKEN unknown EST
1443414_at	3.186	C78513	RIKEN cDNA 5730493B19
1448610_a_at	3.143	Sod2	superoxide dismutase 2, mitochondrial
1417193_at	3.137	Sod2	superoxide dismutase 2, mitochondrial
1451466_at	3.135	D16Ertd472e	DNA segment, Chr 16, ERATO Doi 472, expressed
1451798_at	3.115	Il1rn	interleukin 1 receptor antagonist
1416442_at	3.105	Ier2	immediate early response 2

1417813_at	3.1	Ikbke	inhibitor of kappaB kinase epsilon
1434904_at	3.099	Hivep2	human immunodeficiency virus type I enhancer binding protein 2
1459981_s_at	3.066	C230004D03Rik	AV240926 RIKEN mRNA sequence.
1421236_at	3.064	Ripk2	receptor (TNFRSF)-interacting serine-threonine kinase 2
1435640_x_at	3.049		cDNA clone IMAGE:3414021 mRNA sequence.
1455166_at	3.018	AW146430	ADP-ribosylation factor-like 8
1418191_at	3.01	Usp18	ubiquitin specific protease 18
1419532_at	3.003	Il1r2	interleukin 1 receptor, type II

Table 7.4 Genes uniquely down regulated by treatment with CpG for 0.5 h			
Gene Name	Fold Change	Name	Description
1447803_x_at	6.052	Capg	capping protein (actin filament), gelsolin-like
1437821_at	3.871		RIKEN unknown EST, full insert sequence
1447779_x_at	3.27	Bat1a	BB145254 RIKEN full-length enriched, mRNA sequence.
1458290_at	3.207		Transcribed sequences
1438009_at	3.178	Hist1h2ae	histone 1, H2ae
1428563_at	3.175	Ddx10	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10
1448487_at	3.133	Lrrfip1	leucine rich repeat (in FLII) interacting protein 1
1442710_at	3.123		Transcribed sequences

Table 7.5 Genes downregulated by both CyaA and CyaA with CpG treatment for 0.5 h			
Gene Name	Fold Change	Name	Description
1431212_a_at	4.043	3300001M20R	H3043E11-5 NIA Mouse 15K cDNA Clone mRNA sequence.
1417061_at	3.469	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1

Table 7.6 Genes uniquely down regulated by CyaA treatment for 0.5 h			
Gene Name	Fold Change	Name	Description
1434613_at	7.934	3010002C02Rik	RIKEN cDNA 1810013L24 gene
1423795_at	7.56	Sfpq	splicing factor proline/glutamine rich
1434512_x_at	7.082	Sfrs3	splicing factor, arginine/serine-rich 3 (SRp20)
1448454_at	6.752	Sfrs6	splicing factor, arginine/serine-rich 6
1454805_at	5.529	Wtap	Wilms' tumour 1-associating protein
1430982_at	5.307	Sfrs1	splicing factor, arginine/serine-rich 1 (ASF/SF2)
1416152_a_at	5.106	Sfrs3	splicing factor, arginine/serine-rich 3 (SRp20)
1427691_a_at	4.914	Ifnar2	interferon (alpha and beta) receptor 2
1416106_at	4.854	1110001A12Rik	RIKEN cDNA 0610040B21 gene
1427381_at	4.763	Irg1	immunoresponsive gene 1
1440169_x_at	4.606	Ifnar2	interferon (alpha and beta) receptor 2
1456078_x_at	4.359	4931415M17	BB012080 RIKEN mRNA sequence.
1456117_at	4.355	2600005C20Rik	AV228374 RIKEN mRNA sequence.
1423602_at	4.29	Traf1	Tnf receptor-associated factor 1
1456405_at	4.279	6720461J16Rik	RIKEN cDNA 6720461J16 gene
1426381_at	4.269	Pprc1	cDNA sequence BC013720
1427504_s_at	4.259	Sfrs2	splicing factor, arginine/serine-rich 2 (SC-35)
1418943_at	4.259	AV006891	expressed sequence AV006891
1423796_at	4.248	Sfpq	splicing factor proline/glutamine rich
1436541_at	4.167	2310008H09Rik	RIKEN cDNA 9030624J02 gene
1423192_at	4.164	Pspc1	paraspeckle protein 1
1425498_at	4.076	Prpf4b	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
1435727_s_at	4.029	D15Ertd366e	DNA segment, Chr 15, ERATO Doi 366, expressed
1416928_at	4.017	Rbm12	RNA binding motif protein 12
1433576_at	3.934	Mat2a	methionine adenosyltransferase II, alpha
1428389_s_at	3.91	2610318G08Rik	RIKEN cDNA 2610318G08 gene
1426726_at	3.89	Ppp1r10	protein phosphatase 1, regulatory subunit 10
1428844_a_at	3.877	Bclaf1	BCL2-associated transcription factor 1
1454993_a_at	3.856	Sfrs3	splicing factor, arginine/serine-rich 3 (SRp20)
1422861_s_at	3.803	1110001A05Rik	RIKEN cDNA 1110001A05 gene
1422844_a_at	3.765	2610312E17Rik	RIKEN cDNA 2610312E17 gene
1416150_a_at	3.738	Sfrs3	splicing factor, arginine/serine-rich 3 (SRp20)
1455169_at	3.687	AW558126	RIKEN unknown EST, full insert sequence
1422768_at	3.642	Syncrip	synaptotagmin binding, cytoplasmic RNA interacting protein

1423901_at	3.637	Gtl6	gene trap locus 6
1451174_at	3.62	E430025L02Rik	RIKEN cDNA E430025L02 gene
1452439_s_at	3.611	Sfrs2	splicing factor, arginine/serine-rich 2 (SC-35)
1424750_at	3.608	Zbtb1	zinc finger and BTB domain containing 1
1448133_at	3.605	C87860	expressed sequence C87860
1450685_at	3.6	Arpp19	cAMP-regulated phosphoprotein 19
1436164_at	3.543	C130040I11Rik	solute carrier family 30 (zinc transporter), member 1
1456574_at	3.478	AA407452	BB224546 RIKENmRNA sequence.
1427898_at	3.47	1200013I08Rik	RIKEN cDNA 1200013I08 gene
1451459_at	3.461	Elys	embryonic large molecule derived from yolk sac
1448576_at	3.439	Il7r	interleukin 7 receptor
1425855_a_at	3.427	Crk	v-crk sarcoma virus CT10 oncogene homolog (avian)
1439618_at	3.411	Pde10a	phosphodiesterase 10A
1456415_at	3.368	Zfp451	zinc finger protein 451
1415807_s_at	3.36	Sfrs2	splicing factor, arginine/serine-rich 2 (SC-35)
1419282_at	3.35	Ccl12	chemokine (C-C motif) ligand 12
1429686_at	3.345	3010019O03Rik	RIKEN cDNA 3010019O03 gene
1428845_at	3.338	Bclaf1	BCL2-associated transcription factor 1
1438976_x_at	3.337	Mat2a	gamma-glutamyl carboxylase
1460510_a_at	3.318	1500041J02Rik	RIKEN cDNA 1500041J02 gene
1455173_at	3.297	Gspt1	G1 to phase transition 1
1420951_a_at	3.279	Son	Son cell proliferation protein
1420380_at	3.272	Ccl2	chemokine (C-C motif) ligand 2
1455841_s_at	3.261	Grwd1	Transcribed sequence with WD repeat protein
1456865_x_at	3.25	Rrs1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)
1428487_s_at	3.216	1500041J02Rik	RIKEN cDNA 1500041J02 gene
1416721_s_at	3.194	Sfrs6	splicing factor, arginine/serine-rich 6
1433428_x_at	3.193	Tgm2	transglutaminase 2, C polypeptide
1455171_at	3.186	C630029K18Rik	RIKEN cDNA C630029K18 gene
1435057_x_at	3.182	Paf53	RNA polymerase I associated factor
1451369_at	3.17	Commd5	COMM domain containing 5
1418222_at	3.169	2610024G14Rik	RIKEN cDNA 2610024G14 gene
1416998_at	3.168	Rrs1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)
1438046_at	3.165	AU019823	translocase of inner mitochondrial membrane 8 homolog b (yeast)
1427886_at	3.121	Pom121	RIKEN cDNA 9830109N13 gene
1425933_a_at	3.109	Nt5c2	cDNA clone IMAGE:3487966

1438630_x_at	3.099	Ggcx	AV260654 RIKEN mRNA sequence.
1425956_a_at	3.095	Cdadc1	cytidine and dCMP deaminase domain containing 1
1451680_at	3.093	Npn3	RIKEN cDNA 1700127B04 gene
1455643_s_at	3.089	AW550801	serine racemase
1423045_at	3.085	Ncbp2	nuclear cap binding protein subunit 2
1418255_s_at	3.081	Srf	serum response factor
1428457_at	3.055	5830472M02Rik	RIKEN cDNA 5830472M02 gene
1420093_s_at	3.053	Hnrpdl	heterogeneous nuclear ribonucleoprotein D-like
1440050_at	3.053		Transcribed sequences
1453149_at	3.045	Mftc	mitochondrial folate transporter/carrier
1421529_a_at	3.03	Txnrd1	thioredoxin reductase 1
1428629_at	3.029	6330417C12Rik	RIKEN cDNA 2810401C22 gene
1432995_at	3.023	2900057E15Rik	Adult male hippocampus cDNA, RIKEN
1426813_at	3.022	2610020N02Rik	RIKEN cDNA 2610020N02 gene
1437238_x_at	3.011	C87860	expressed sequence C87860

Table 7.7 Genes uniquely downregulated by co-treatment with CyaA and CpG for 0.5 h			
Gene Name	Fold Change	Name	Description
1429499_at	9.434	Fbxo5	F-box only protein 5
1417061_at	8.552	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1
1448760_at	6.139	Zfp68	Zinc finger protein 68
1457246_at	5.701	G430067P06Rik	AU040322 , mRNA sequence.
1448412_a_at	5.378	0610009M14Rik	RIKEN cDNA 0610009M14 gene
1448566_at	5.325	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1
1424143_a_at	5.278	Ris2	retroviral integration site 2
1424144_at	5.252	Ris2	retroviral integration site 2
1447227_at	5.241		Transcribed sequences
1437716_x_at	5.172	Kif22	kinesin family member 22
1455648_at	5.157		Similar to zinc finger protein 91 (HPF7, HTF10) (LOC278757), mRNA
1454609_x_at	5.123	6430527G18Rik	RIKEN cDNA 6430527G18 gene
1449060_at	4.44	Kif2c	kinesin family member 2C
1451382_at	4.411	1810008K03Rik	RIKEN cDNA 1810008K03 gene
1460247_a_at	4.383	Skp2	AV259620 RIKEN mRNA sequence.
1445626_at	4.357		Transcribed sequences
1428479_at	4.286	Nfatc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
1415997_at	4.175	Txnip	thioredoxin interacting protein
1431212_a_at	4.109	3300001M20Rik	H3043E11-5 mRNA sequence.
1436944_x_at	4.069	9030221M09Rik	Transcribed sequence
1438234_at	4.066	Wdr26	WD repeat domain 26
1434907_at	3.914	Tbpl1	solute carrier family 2, member 12
1422535_at	3.84	Ccne2	cyclin E2
1437072_at	3.823	Arhgap25	RIKEN cDNA A130039I20 gene
1454746_at	3.75	Plekhm1	cDNA sequence BC038943
1438931_s_at	3.749	Sesn1	sestrin 1
1450899_at	3.736	Nedd1	neural precursor cell expressed, developmentally down-regulated gene 1
1428083_at	3.722	2310043N10Rik	Adult male cecum cDNA, RIKEN unknown EST
1452948_at	3.704	1810019A08Rik	RIKEN cDNA 1810019A08 gene
1438010_at	3.681	AV028368	expressed sequence AV028368
1418380_at	3.644	Terf1	telomeric repeat binding factor 1
1442155_at	3.621	4632427E13Rik	RIKEN cDNA 4632427E13 gene
1429758_at	3.535	1700017B05Rik	RIKEN cDNA 1700017B05 gene
1439650_at	3.463	Rtn4	16 days embryo head cDNA, RIKEN unknown EST

1419853_a_at	3.451		Transcribed sequences
1456479_at	3.444	4732481H14Rik	RIKEN cDNA 4732481H14 gene
1416118_at	3.44	2310035M22Rik	RIKEN cDNA 2310035M22 gene
1427080_at	3.435	2610036D13Rik	RIKEN cDNA 2610036D13 gene
1455585_at	3.43	3110001H15Rik	RIKEN cDNA 3110001H15 gene
1442786_s_at	3.408	D5Bwg0860e	DNA segment, Chr 5, Brigham & Women's Genetics 0860 expressed
1437626_at	3.381		Brf2 gene, 3' UTR
1439482_at	3.375	1110020K19Rik	Activated spleen cDNA, RIKEN CDNA
1415715_at	3.374	2410015J15Rik	stem-loop binding protein
1416773_at	3.294	Wee1	wee 1 homolog (S. pombe)
1416915_at	3.288	Msh6	mutS homolog 6 (E. coli)
1437236_a_at	3.278		AV263718 RIKEN mRNA sequence.
1434348_at	3.277	Fez2	fasciculation and elongation protein zeta 2 (zygin II)
1431316_at	3.276	Itch	itchy
1426614_at	3.264	3632413B07Rik	RIKEN cDNA 3632413B07 gene
1434079_s_at	3.238	Mcm2	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)
1419513_a_at	3.223	Ect2	ect2 oncogene
1430805_s_at	3.221	4932432N11Rik	RIKEN cDNA 4932432N11 gene
1437431_at	3.218		Transcribed sequences
1429345_at	3.218	D2Ertd435e	cDNA, RIKEN full-length enriched library, clone
1436986_at	3.217	Sntb2	syntrophin, basic 2
1455092_at	3.214	Zfp207	zinc finger protein 207
1459360_at	3.205		Transcribed sequence
1449217_at	3.204	Casp8ap2	caspase 8 associated protein 2
1452276_at	3.195	Smarcad1	matrix-associated actin-dependent regulator of chromatin
1440083_at	3.179	A430061O12Rik	0 day neonate thymus cDNA, RIKEN
1449947_s_at	3.178	Atbf1	AT motif binding factor 1
1444728_at	3.173		Transcribed sequences
1443733_x_at	3.156	Pold3	polymerase (DNA-directed), delta 3, accessory subunit
1429155_at	3.153	4933411K20Rik	RIKEN cDNA 4933411K20 gene
1415789_a_at	3.138	BC002236	cDNA sequence BC002236
1436330_x_at	3.136	6.72E+21	Similar to zinc finger protein 91 (HPF7, HTF10) (LOC278757), mRNA
1452722_a_at	3.133	Cul5	cullin 5
1418969_at	3.126	Skp2	AV259620 RIKEN full-length mRNA sequence.
1442827_at	3.109		toll-like receptor 4
1439722_at	3.101		unknown EST,

1426752_at	3.096	Phf17	PHD finger protein 17
1460548_a_at	3.095	Eral1	Era (G-protein)-like 1 (E. coli)
1434767_at	3.086	C79407	expressed sequence C79407
1426536_at	3.073	Narg2	NMDA receptor-regulated gene 2
1456341_a_at	3.052	Bteb1	AV354744 RIKEN mRNA sequence.
1454923_at	3.033	AW214353	Transcribed sequence RIKEN cDNA 5730493B19
1418546_a_at	3.03	1700095N21Rik	RIKEN cDNA 1700095N21 gene
1459843_s_at	3.011	Smad1	MAD homolog 1 (Drosophila)
1442254_at	3		Transcribed sequences

Table 7.8 Genes uniquely upregulated by co-treatment with CyaA and CpG for 6 h			
Gene Name	Fold Change	Name	Description
1448183_a_at	6.627	Hif1a	hypoxia inducible factor 1, alpha subunit
1438115_a_at	6.081	Slc9a3r1	BB805362 RIKEN mRNA sequence.
1448793_a_at	5.793	Sdc4	syndecan 4
1451458_at	5.672	Tmem2	transmembrane protein 2
1450982_at	5.3	Slc9a3r1	solute carrier family 9, isoform 3 regulator 1
1416035_at	5.273	Hif1a	hypoxia inducible factor 1, alpha subunit
1435222_at	5.229	Foxp1	forkhead box P1
1436555_at	5.221	AI158848	solute carrier family 7 member 2
1433920_at	5.034	Sema4c	sema domain, immunoglobulin domain (Ig) (semaphorin) 4C
1421142_s_at	4.89	Foxp1	forkhead box P1
1428306_at	4.852	Ddit4	DNA-damage-inducible transcript 4
1426501_a_at	4.757	T2bp	Traf2 binding protein
1424711_at	4.682	Tmem2	transmembrane protein 2
1437763_at	4.679	BC030335	cDNA sequence BC030335
1419655_at	4.544	Tle3	transducin-like enhancer of split 3, homolog of Drosophila E(spl)
1452160_at	4.458	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase
1423289_a_at	4.406	1810029B16Rik	RIKEN cDNA 1810029B16 gene
1434828_at	4.36	1600010D10Rik	hypothetical protein B430201A12
1417519_at	4.26	Plagl2	pleiomorphic adenoma gene-like 2
1434432_at	4.185	1700051E09Rik	ligase III, DNA, ATP-dependent
1423006_at	4.173	Pim1	proviral integration site 1
1457213_a_at	4.006	Dgkh	12 days embryo spinal ganglion cDNA, RIKEN full insert sequence
1447100_s_at	3.982	5730508B09Rik	RIKEN cDNA 5730508B09 gene
1425023_at	3.96	Usp3	ubiquitin specific protease 3
1435221_at	3.915	Foxp1	forkhead box P1
1421457_a_at	3.907	Samsn1	SAM domain, SH3 domain and nuclear localisation signals, 1
1449454_at	3.9	Bst1	bone marrow stromal cell antigen 1
1449222_at	3.862	Ebi3	Epstein-Barr virus induced gene 3
1434015_at	3.815	Slc2a6	solute carrier family 2 (facilitated glucose transporter), member 6
1450459_at	3.797	2010106G01Rik	RIKEN cDNA 2010106G01 gene
1460466_at	3.787	1700047I17Rik	RIKEN cDNA 1700047I17 gene
1421140_a_at	3.725	Foxp1	forkhead box P1
1435872_at	3.707	Pim1	proviral integration site 1
1433617_s_at	3.647	B4galt5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5

1434438_at	3.619	Samhd1	SAM domain and HD domain, 1
1418201_at	3.608	AI194308	expressed sequence AI194308
1449645_s_at	3.558	Cct3	chaperonin subunit 3 (gamma)
1460337_at	3.552	Sh3kbp1	SH3-domain kinase binding protein 1
1455332_x_at	3.544	Fcgr2b	Fc receptor, IgG, low affinity IIb
1454666_at	3.53	Klf3	Kruppel-like factor 3 (basic)
1420635_a_at	3.515	Tcirg1	T-cell, immune regulator 1
1423596_at	3.508	Nek6	NIMA (never in mitosis gene a)-related expressed kinase 6
1435137_s_at	3.481		BB119177 RIKEN mRNA sequence.
1426541_a_at	3.471	2310067E08Rik	RIKEN cDNA 2310067E08 gene
1429335_at	3.471	Snapc1	small nuclear RNA activating complex, polypeptide 1
1448830_at	3.47	Dusp1	dual specificity phosphatase 1
1426721_s_at	3.47	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase
1435133_at	3.438	Ugcg	UDP-glucose ceramide glucosyltransferase
1449628_s_at	3.433	Stard7	START domain containing 7
1419068_at	3.385	Rabgef1	RAB guanine nucleotide exchange factor (GEF) 1
1424625_a_at	3.372	6030446I19Rik	RIKEN cDNA 6030446I19 gene
1448380_at	3.348	Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein
1429678_at	3.346	5730508B09Rik	RIKEN cDNA 5730508B09 gene
1426540_at	3.336	2310067E08Rik	RIKEN cDNA 2310067E08 gene
1453238_s_at	3.333	3930401B19Rik	AU018141 Mouse mRNA sequence.
1421392_a_at	3.327	Birc3	baculoviral IAP repeat-containing 2
1419069_at	3.327	Rabgef1	RAB guanine nucleotide exchange factor (GEF) 1
1449124_at	3.318	Rgl1	ral guanine nucleotide dissociation stimulator,-like 1
1417300_at	3.299	Smpdl3b	acid sphingomyelinase-like phosphodiesterase
1432478_a_at	3.269	4930534K13Rik	RIKEN cDNA 4930534K13 gene
1450850_at	3.266	Vil2	villin 2
1451988_s_at	3.257	2010012F05Rik	RIKEN cDNA 2010012F05 gene
1419355_at	3.245	Klf7	Kruppel-like factor 7 (ubiquitous)
1434555_at	3.24	Anp32a	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
1460430_at	3.231	Rap2c	RAP2C, member of RAS oncogene family
1426716_at	3.227	Tdrd7	RIKEN cDNA 5730495N10 gene
1449507_a_at	3.219	Cd47	CD47 antigen(Rh-related antigen, integrin-associated signal transducer)
1417189_at	3.215	Psme2	proteasome (prosome, macropain) 28 subunit, beta
1452161_at	3.155	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase
1423757_x_at	3.149	Igfbp4	insulin-like growth factor binding protein 4

1416708_a_at	3.127	D7Bwg0611e	DNA segment, Chr 7, Brigham & Women's Genetics 0611 expressed
1425193_at	3.111	2010106G01Rik	RIKEN cDNA 2010106G01 gene
1438097_at	3.109	Rab20	RAB20, member RAS oncogene family
1458299_s_at	3.092	Nfkbie	BB820441 RIKENmRNA sequence.
1416122_at	3.09	Ccnd2	cyclin D2
1451466_at	3.089	D16Ertd472e	DNA segment, Chr 16, ERATO Doi 472, expressed
1418854_at	3.082	Birc2	baculoviral IAP repeat-containing 3
1419267_at	3.077	Nfyb	nuclear transcription factor-Y beta
1423756_s_at	3.072	Igfbp4	insulin-like growth factor binding protein 4
1456498_at	3.049		integrin alpha 4
1430752_at	3.045	C330006D17Rik	BB397948 RIKEN mRNA sequence.
1455242_at	3.034	Foxp1	forkhead box P1
1451941_a_at	3.021	Fcgr2b	Fc receptor, IgG, low affinity IIb
1458668_at	3.008	Tpd52	tumor protein D52
1424574_at	3.005	4432412D15Rik	RIKEN cDNA 3110020O18 gene
1435640_x_at	3.003		uv85h11.x1 Soares mouse 3NbMS mRNA sequence.

Table 7.9 Genes uniquely upregulated by CyaA treatment for 6 h			
Gene Name	Fold Change	Name	Description
1418825_at	6.391	Ifi1	interferon inducible protein 1
1448890_at	5.928	Klf2	Kruppel-like factor 2 (lung)
1451567_a_at	5.662	Ifi203	interferon activated gene 203
1448958_at	5.503	Ifrg15	interferon alpha responsive gene
1449037_at	5.35	Crem	cAMP responsive element modulator
1422781_at	5.328	Tlr3	toll-like receptor 3
1418115_s_at	4.964	Ifrg15	interferon alpha responsive gene
1422782_s_at	4.952	Tlr3	toll-like receptor 3
1437176_at	4.948	AI451557	Transcribed sequences
1429251_at	4.733	Prdm2	RIKEN cDNA 4833427P12 gene (4833427P12Rik), mRNA
1418322_at	4.634	Crem	cAMP responsive element modulator
1422631_at	4.539	Ahr	aryl-hydrocarbon receptor
1435208_at	4.524		Transcribed sequence RIKEN cDNA 5730493B19
1436120_at	4.414		RIKEN full-length enriched library, clone:F630035N16
1423566_a_at	4.402	Hsp105	heat shock protein 105
1418131_at	4.355	Samhd1	SAM domain and HD domain, 1
1434401_at	4.354		zinc finger, CCHC domain containing 2
1417372_a_at	4.348	Peli1	pellino 1
1448775_at	4.323	Ifi203	interferon activated gene 203
1458365_at	4.233	AI467484	expressed sequence AI467484
1460603_at	4.187	AA175286	Transcribed sequences
1416397_at	4.003	Mesdc1	mesoderm development candidate 1
1448757_at	3.971	Pml	promyelocytic leukemia
1424727_at	3.931	Ccr5	chemokine (C-C motif) receptor 5
1423838_s_at	3.915	2400003C14Rik	RIKEN cDNA 2400003C14 gene
1434566_a_at	3.904	4732496O08Rik	RIKEN cDNA 4732496O08 gene
1433428_x_at	3.904	Tgm2	transglutaminase 2, C polypeptide
1425993_a_at	3.859	Hsp105	heat shock protein 105
1418219_at	3.835	Il15	interleukin 15
1422615_at	3.804	Map4k4	mitogen-activated protein kinase kinase kinase kinase 4
1447849_s_at	3.799	Maf	AV323441 RIKEN mRNA sequence.
1417371_at	3.791	Peli1	pellino 1
1423071_x_at	3.779	Itga6	CDNA clone MGC:73493 IMAGE:6813048, complete cds
1416151_at	3.776	Sfrs3	splicing factor, arginine/serine-rich 3 (SRp20)

1420710_at	3.755	Rel	reticuloendotheliosis oncogene
1429564_at	3.75	5830443C21Rik	RIKEN cDNA 5830443C21 gene
1436899_at	3.738	2700019D07Rik	RIKEN cDNA 2700019D07 gene
1423520_at	3.703	Lmnb1	lamin B1
1426903_at	3.688	Fndc3	DNA segment, Chr 14, ERATO Doi 453, expressed
1422141_s_at	3.681	Csprs	component of Sp100-rs
1449176_a_at	3.679	Dck	deoxycytidine kinase
1426471_at	3.67	Zfp52	zinc finger protein 52
1455900_x_at	3.656	Tgm2	BB041811 RIKEN mRNA sequence.
1432459_a_at	3.634	Rog	repressor of GATA
1434580_at	3.63	Enpp4	ectonucleotide pyrophosphatase/phosphodiesterase 4
1426942_at	3.627	Aim1	absent in melanoma 1
1450853_at	3.624	Tle4	transducin-like enhancer of split 4, homolog of Drosophila E(spl)
1418772_at	3.614	BC016423	cDNA sequence BC016423
1435526_at	3.604	A130072J07	interferon alpha responsive gene
1434304_s_at	3.564	D10Ertd438e	DNA segment, Chr 10, ERATO Doi 438, expressed
1449875_s_at	3.548	H2-T10	histocompatibility 2, T region locus 10
1439276_at	3.539		Transcribed sequence RIKEN cDNA 5730493B19 [Mus musculus]
1437277_x_at	3.499	Tgm2	transglutaminase 2, C polypeptide
1424929_a_at	3.489	Trim26	tripartite motif protein 26
1450454_at	3.475	Tor3a	torsin family 3, member A
1434285_at	3.418	2700017I06Rik	RIKEN cDNA 2700017I06 gene (2700017I06Rik), mRNA
1452741_s_at	3.386	Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial
1436562_at	3.377	Ddx58	RIKEN cDNA 6430573D20 gene
1443858_at	3.364	Trim34	RIKEN cDNA 9230105E10 gene
1423837_at	3.355	2400003C14Rik	RIKEN cDNA 2400003C14 gene
1422006_at	3.344	Prkr	protein kinase, interferon-inducible double stranded RNA dependent
1428838_a_at	3.331	Dck	deoxycytidine kinase
1418077_at	3.296	Trim21	tripartite motif protein 21
1428715_at	3.293	2810423A18Rik	glutamine fructose-6-phosphate transaminase 1
1419914_s_at	3.284	D10Ertd438e	DNA segment, Chr 10, ERATO Doi 438, expressed
1436172_at	3.263		cDNA full insert sequence
1421298_a_at	3.25	Hipk1	homeodomain interacting protein kinase 1
1427705_a_at	3.249	Nfkb1	nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
1456574_at	3.248	AA407452	BB224546 RIKEN mRNA sequence.
1451122_at	3.239	Idi1	isopentenyl-diphosphate delta isomerase

1433454_at	3.192	AW539457	expressed sequence AW539457
1437663_at	3.189	D430024F16Rik	RIKEN cDNA D430024F16 gene
1419532_at	3.186	Il1r2	interleukin 1 receptor, type II
1417601_at	3.186	Rgs1	regulator of G-protein signaling 1
1439618_at	3.164	Pde10a	phosphodiesterase 10A
1433514_at	3.163	Etnk1	ethanolamine kinase 1
1422005_at	3.157	Prkr	protein kinase, interferon-inducible double stranded RNA dependent
1440342_at	3.15		BB276544 RIKEN mRNA sequence.
1426862_at	3.148	9130023F12Rik	RIKEN cDNA 9130023F12 gene
1418116_at	3.145	Ifrg15	interferon alpha responsive gene
1419879_s_at	3.13	Trim25	tripartite motif protein 25
1459151_x_at	3.112	Ifi35	interferon-induced protein 35
1443673_x_at	3.105		BB710847 RIKEN full-length mRNA sequence.
1456358_at	3.102	Etv3	ets variant gene 3
1424417_at	3.096	2210415M20Rik	RIKEN cDNA 2210415M20 gene
1443159_at	3.095	9130221J17Rik	thioredoxin reductase 1
1455161_at	3.095	AI504432	expressed sequence AI504432
1460207_s_at	3.074	E2f5	E2F transcription factor 5
1421998_at	3.073	Tor3a	torsin family 3, member A
1419272_at	3.066	Myd88	myeloid differentiation primary response gene 88
1423804_a_at	3.063	Idi1	isopentenyl-diphosphate delta isomerase
1440011_at	3.062		cDNA, RIKEN full-length full insert sequence
1422698_s_at	3.039	Jarid2	jumonji, AT rich interactive domain 2
1421733_a_at	3.038	Tpst1	protein-tyrosine sulfotransferase 1
1419609_at	3.034	Ccr1	chemokine (C-C motif) receptor 1
1428323_at	3.018	Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial
1434567_at	3.012	4732496O08Rik	RIKEN cDNA 4732496O08 gene
1428762_at	3.009	4921526G09Rik	RIKEN cDNA 4921526G09 gene
1438004_at	3.006	Pols	polymerase (DNA directed) sigma

Table 7.10 Genes uniquely upregulated by CpG treatment for 6 h			
Gene Name	Fold Change	Name	Description
1444402_at	9.575	C230027N18Rik	RIKEN cDNA C230027N18 gene
1450413_at	6.622	Pdgfb	platelet derived growth factor, B polypeptide
1416998_at	6.131	Rrs1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)
1421408_at	5.447	Igsf6	immunoglobulin superfamily, member 6
1423543_at	5.029	Swap70	SWAP complex protein
1419282_at	4.888	Ccl12	chemokine (C-C motif) ligand 12
1435277_x_at	4.858	Nme1	AV156640 mRNA sequence.
1442434_at	4.734	D8Ertd82e	DNA segment, Chr 8, ERATO Doi 82, expressed
1459571_at	4.33		Transcribed sequences
1449519_at	4.215	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha
1418020_s_at	4.212	Cpd	carboxypeptidase D
1449363_at	4.21	Atf3	activating transcription factor 3
1450643_s_at	4.146	Acsl1	fatty acid Coenzyme A ligase, long chain 2
1424290_at	4.119	BC010311	cDNA sequence BC010311
1417065_at	4.111	Egr1	early growth response 1
1454806_at	4.065	D12Ertd553e	DNA segment, Chr 12, ERATO Doi 553, expressed
1435626_a_at	4.05	Herpud1	homocysteine-inducible, endoplasmic reticulum stress-inducible
1423267_s_at	4.027	Itga5	integrin alpha 5 (fibronectin receptor alpha)
1434225_at	3.989	Swap70	SWAP complex protein
1422978_at	3.898	Cybb	cytochrome b-245, beta polypeptide
1440809_at	3.855		BB530868 RIKEN mRNA sequence.
1447519_x_at	3.748	2610005B21Rik	H3118A08-3 mRNA sequence.
1436026_at	3.746	1110032O19Rik	unknown EST, full insert sequence
1459853_x_at	3.671		AV312901 RIKEN mRNA sequence.
1460620_at	3.63	Zfp592	RIKEN cDNA A730014M16 gene
1450414_at	3.578	Pdgfb	platelet derived growth factor, B polypeptide
1435333_at	3.557	1110007M04Rik	RIKEN cDNA 1110007M04 gene
1454815_at	3.442	Sertad2	SERTA domain containing 2
1430623_s_at	3.402	5830411E10Rik	RIKEN cDNA 5830411E10 gene
1451767_at	3.393	Ncf1	601218843F1 IMAGE:3587723 mRNA sequence.
1459219_at	3.375		RIKEN full-length unknown EST
1423614_at	3.335	E430036I04Rik	BB329408 RIKEN full-length mRNA sequence.
1416313_at	3.316	AI839562	expressed sequence AI839562
1437100_x_at	3.314	Pim3	BB206220 RIKEN full-length mRNA sequence.

1437917_at	3.308	D530037H12Rik	cDNA, RIKEN full-length
1460699_at	3.287	Rps27	ribosomal protein S27
1417523_at	3.267	Plek	pleckstrin
1428172_at	3.26	Prpf39	PRP39 pre-mRNA processing factor 39 homolog (yeast)
1455665_at	3.238		Transcribed sequences
1428637_at	3.234	1810038L18Rik	Similar to DYRK2 protein (LOC380662), mRNA
1455300_at	3.234	E130014J05Rik	RIKEN cDNA E130014J05 gene
1417801_a_at	3.216	Ppfibp2	protein tyrosine phosphatase, receptor-type, F interacting protein
1448898_at	3.216	Ccl9	chemokine (C-C motif) ligand 9
1454109_a_at	3.207	Ptdsr	phosphatidylserine receptor
1452858_at	3.2	Elavl1	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
1418092_s_at	3.197	Trip10	thyroid hormone receptor interactor 10
1456163_at	3.187	2700049P18Rik	RIKEN cDNA 2700049P18 gene
1420509_at	3.164	2810036K01Rik	RIKEN cDNA 2810036K01 gene
1425609_at	3.16	Ncf1	601218843F1 mRNA sequence.
1429490_at	3.148	D2Ertd145e	RIKEN cDNA 6530403D07 gene
1450764_at	3.131	Aoah	acyloxyacyl hydrolase
1424289_at	3.124	BC010311	cDNA sequence BC010311
1448185_at	3.116	Herpud1	homocysteine-inducible, endoplasmic reticulum stress-inducible
1425035_s_at	3.097	Dnmt3l	DNA (cytosine-5-)-methyltransferase 3-like
1427518_at	3.096	D10627	5' similar to Mus musculus DNA-binding protein mRNA, mRNA seq
1433851_at	3.093	Ppp4r2	protein phosphatase 4, regulatory subunit 2
AFFX-TransRecM	3.082	Tfrc	
1452661_at	3.062	Tfrc	transferrin receptor
1436511_at	3.059	BC031781	cDNA sequence BC031781
1452833_at	3.052	Rapgef2	Rap guanine nucleotide exchange factor (GEF) 2
1441973_at	3.04	Zfp295	BB091040 RIKEN full-length mRNA sequence.
1447775_x_at	3.029	Zfp622	BB278135 RIKEN full-length mRNA sequence.

Table 7.11 Genes uniquely down regulated by co-treatment with CyaA and CpG for 6 h			
Gene Name	Fold Change	Name	Description
1457753_at	8.464	Tlr13	Similar to toll-like receptor (LOC279572), mRNA
1417420_at	6.472	Ccnd1	cyclin D1
1417419_at	6.454	Ccnd1	cyclin D1
1425584_x_at	5.946		Mus musculus cDNA clone MGC:6728 IMAGE:3588654, complete cds.
1454742_at	5.869	Rasgef1b	RasGEF domain family, member 1B
1448955_s_at	5.81	Cadps	Ca<2+>dependent activator protein for secretion
1424443_at	5.797	Tm6sf1	transmembrane 6 superfamily member 1
1448698_at	5.648	Ccnd1	cyclin D1
1453069_at	5.487	Pik3cb	phosphatidylinositol 3-kinase, catalytic, beta polypeptide
1449061_a_at	5.222	Prim1	DNA primase, p49 subunit
1443894_at	5.098	Evi2a	ecotropic viral integration site 2a
1435059_at	4.823	AV239055	Transcribed sequence with moderate similarity to protein NP_003878.1
1424356_a_at	4.793	BC019776	cDNA sequence BC019776
1428834_at	4.728	Dusp4	dual specificity phosphatase 4
1448113_at	4.696	Stmn1	stathmin 1
1417327_at	4.687	Cav2	caveolin 2
1448664_a_at	4.682	Apeg1	aortic preferentially expressed gene 1
1419315_at	4.66	Slamf9	SLAM family member 9
1419172_at	4.646	Dhfr	dihydrofolate reductase
1451867_x_at	4.632	Arhgap6	Rho GTPase activating protein 6
1428444_at	4.601	Asb2	ankyrin repeat and SOCS box-containing protein 2
1416299_at	4.528	Shcbp1	Shc SH2-domain binding protein 1
1425815_a_at	4.518	Hmmr	hyaluronan mediated motility receptor (RHAMM)
1422747_at	4.406	Chek2	CHK2 checkpoint homolog (S. pombe)
1455227_at	4.396	B230106I24Rik	RIKEN cDNA B230106I24 gene
1456307_s_at	4.391	Adcy7	adenylate cyclase 7
1428479_at	4.304	Nfatc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
1428351_at	4.262	2810423O19Rik	RIKEN cDNA 2810423O19 gene
1436513_at	4.257	3526402J09Rik	RIKEN cDNA 3526402J09 gene
1423465_at	4.246	Sdfr2	stromal cell derived factor receptor 2
1428522_at	4.239	Ttf2	0 day neonate lung cDNA full insert sequence
1415855_at	4.212	Kitl	kit ligand
1421027_a_at	4.206	Mef2c	myocyte enhancer factor 2C
1424118_a_at	4.184	2600017H08Rik	RIKEN cDNA 2600017H08 gene

1426473_at	4.163	Dnajc9	DnaJ (Hsp40) homolog, subfamily C, member 9
1417938_at	4.102	Rad51ap1	RAD51 associated protein 1
1424852_at	4.096	5430401D19Rik	myocyte enhancer factor 2C
1452242_at	4.087	1200008O12Rik	RIKEN cDNA 1200008O12 gene
1457266_at	4.074	AW322671	Transcribed sequences
1450069_a_at	4.057	Cugbp2	BB667096 RIKEN mRNA sequence.
1422637_at	4.05	Rassf5	Ras association (RalGDS/AF-6) domain family 5
1448938_at	4.018	Rpa3	replication protein A3
1450700_at	4.006	Cdc42ep3	CDC42 effector protein (Rho GTPase binding) 3
1426915_at	3.958	Dapk1	death associated protein kinase 1
1421205_at	3.924	Atm	ataxia telangiectasia mutated homolog (human)
1451353_at	3.916	Tm6sf1	transmembrane 6 superfamily member 1
1451154_a_at	3.913	Cugbp2	hypothetical protein B230218O03
1417122_at	3.905	Vav3	vav 3 oncogene
1424683_at	3.868	1810015C04Rik	RIKEN cDNA 1810015C04 gene
1436368_at	3.864	Slc16a10	BB140436 RIKEN full-length mRNA sequence.
1417995_at	3.84	Ptpn8	protein tyrosine phosphatase, non-receptor type 8
1422444_at	3.831	Itga6	integrin alpha 6
1424125_at	3.814	Kcnk13	potassium channel, subfamily K, member 13
1441962_at	3.778	F730011J02	hypothetical protein F730011J02
1434856_at	3.737	A130096K20	hypothetical protein A130096K20
1438852_x_at	3.727	Mcm6	minichromosome maintenance deficient 6
1445882_at	3.704		Transcribed sequences
1437069_at	3.686	D330025H14Rik	oxysterol binding protein-like 8
1422659_at	3.682	Camk2d	calcium/calmodulin-dependent protein kinase II, delta
1436193_at	3.68	Man1c1	mannosidase, alpha, class 1C, member 1
1435745_at	3.657	5031439G07Rik	RIKEN cDNA 5031439G07 gene
1426397_at	3.652	Tgfbr2	transforming growth factor, beta receptor II
1455849_at	3.622	Nav1	neuron navigator 1
1443208_at	3.605		unknown EST, full insert sequence
1415834_at	3.588	Dusp6	dual specificity phosphatase 6
1450881_s_at	3.584	Tm7sf1	Mus musculus ES cells cDNA, RIKEN full-length
1449699_s_at	3.573	C330027C09Rik	RIKEN cDNA C330027C09 gene
1426801_at	3.558	Sep-08	septin 8
1418763_at	3.546	D16Ertd502e	DNA segment, Chr 16, ERATO Doi 502, expressed
1427763_a_at	3.536	Camk2d	calcium/calmodulin-dependent protein kinase II, delta

1450626_at	3.528	Manba	mannosidase, beta A, lysosomal
1428480_at	3.525	D4Ertd421e	DNA segment, Chr 4, ERATO Doi 421, expressed
1434135_at	3.522	D230016N13Rik	RIKEN cDNA D230016N13 gene
1427197_at	3.515	Atr	Mus musculus protein kinase ATR (Atr) mRNA, partial cds.
1434472_at	3.514	5031436O03Rik	RIKEN EST, full insert sequence
1430574_at	3.513	Cdkn3	cyclin-dependent kinase inhibitor 3
1426739_at	3.509	Donson	downstream neighbor of SON
1444040_at	3.508	Lair1	DNA segment, Chr 7, Brigham & Women's Genetics 0421 expressed
1450065_at	3.459	Adcy7	adenylate cyclase 7
1424328_s_at	3.446	3200002M19Rik	RIKEN cDNA 3200002M19 gene
1452917_at	3.444	Rfc5	replication factor C (activator 1) 5
1424396_a_at	3.439	Asrgl1	asparaginase like 1
1425058_at	3.434	Zfp472	zinc finger protein 472
1434131_at	3.424	Rufy1	RUN and FYVE domain containing 1
1415849_s_at	3.416	Stmn1	stathmin 1
1433649_at	3.401	Aof1	amine oxidase, flavin containing 1
1428527_at	3.401	Snx7	sorting nexin 7
1448318_at	3.365	Adfp	adipose differentiation related protein
1436756_x_at	3.359	Hadhsc	BB114220 RIKEN full-length mRNA sequence.
1424629_at	3.348	Brca1	breast cancer 1
1419833_s_at	3.347	E030006K04Rik	RIKEN cDNA E030006K04 gene
1436339_at	3.343	1810058I24Rik	RIKEN full-length unknown EST, full insert sequence
1460695_a_at	3.297	2300006M17Rik	RIKEN cDNA 2300006M17 gene
1453266_at	3.295	9230111I22Rik	RIKEN cDNA 2310026P19 gene
1418369_at	3.293	Prim1	DNA primase, p49 subunit
1423680_at	3.286	Fads1	RIKEN cDNA 0710001O03 gene
1437520_a_at	3.28	Pcnt2	pericentrin 2
1418289_at	3.278	Nes	nestin
1417116_at	3.278	Slc6a8	solute carrier family 6(neurotransmitter transporter,creatine),member 8
1449116_a_at	3.262	Dtymk	deoxythymidylate kinase
1436907_at	3.257	Nav1	neuron navigator 1
1436584_at	3.246	Spry2	sprouty homolog 2 (Drosophila)
1452283_at	3.242	AW123240	expressed sequence AW123240
1424069_at	3.233	Napg	N-ethylmaleimide sensitive fusion protein attachment protein gamma
1423063_at	3.227	Dnmt3a	BB795491 RIKEN full-length enriched mRNA sequence.
1428988_at	3.219	Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3

1430332_a_at	3.218	Gus	beta-glucuronidase
1417704_a_at	3.21	Arhgap6	Rho GTPase activating protein 6
1434403_at	3.207	Spred2	sprouty protein with EVH-1 domain 2, related sequence
1422535_at	3.197	Ccne2	cyclin E2
1443534_at	3.196		Transcribed sequences
1416254_a_at	3.192	Vps16	vacuolar protein sorting 16 (yeast)
1415878_at	3.177	Rrm1	ribonucleotide reductase M1
1419217_at	3.167	Gnefr	guanine nucleotide exchange factor (RCC1 related)
1452714_at	3.157	1200003E16Rik	RIKEN cDNA 1200003E16 gene
1417394_at	3.142	Klf4	Kruppel-like factor 4 (gut)
1430526_a_at	3.137	Smarca2	SWI/SNF related, matrix associated, actin dependent reg of chromatin
1455359_at	3.112	C130080N23Rik	16 days embryo head cDNA, RIKEN EST, full insert sequence
1433564_at	3.099	Dgkd	diacylglycerol kinase, delta
1439034_at	3.076	Spn	BB160586 RIKEN mRNA sequence.
1439067_at	3.071	Lair1	DNA segment, Chr 7, Brigham & Women's Genetics 0421 expressed
1421366_at	3.051	Clecsf5	C-type lectin, superfamily member 5
1439616_at	3.046	AV026040	Transcribed sequence e
1419153_at	3.035	2810417H13Rik	RIKEN cDNA 2810417H13 gene
1429058_at	3.019	1110004B13Rik	RIKEN cDNA 1110004B13 gene
1416904_at	3.007	Mbnl1	muscleblind-like 1 (Drosophila)
1440715_s_at	3.006	D11Ertd497e	DNA segment, Chr 11, ERATO Doi 497, expressed

Table 7.12 Genes uniquely downregulated by treatment with CpG for 6 h			
Gene Name	Fold Change	Name	Description
1451206_s_at	37.31	Pscdbp	pleckstrin homology, Sec7 and coiled-coil domains, binding protein
1435697_a_at	18.4	Pscdbp	BB503614 RIKEN mRNA sequence.
1438619_x_at	6.522	Zdhhc14	zinc finger, DHHC domain containing 14
1438975_x_at	6.384	Zdhhc14	AV361868 RIKEN mRNA sequence.
1418183_a_at	6.318	Pscd1	pleckstrin homology, Sec7 and coiled-coil domains 1
1437614_x_at	6.196	Zdhhc14	AV223474 RIKEN full-length mRNA sequence.
1436512_at	5.552	A630084M22Rik	RIKEN cDNA A630084M22 gene
1450997_at	5.216	Stk17b	serine/threonine kinase 17b (apoptosis-inducing)
1423100_at	5.019	Fos	FBJ osteosarcoma oncogene
1454617_at	4.946	Arrdc3	arrestin domain containing 3
1418300_a_at	4.911	Mknk2	MAP kinase-interacting serine/threonine kinase 2
1416926_at	4.76	Trp53inp1	transformation related protein 53 inducible nuclear protein 1
1438156_x_at	4.742	Cpt1a	BB119196 RIKEN mRNA sequence.
1424987_at	4.677	5430435G22Rik	RIKEN cDNA 5430435G22 gene
1448830_at	4.203	Dusp1	dual specificity phosphatase 1
1449037_at	4.092	Crem	cAMP responsive element modulator
1423269_a_at	4.046	Nedd4l	developmentally down-regulated gene 4-like
1450750_a_at	4.042	Nr4a2	nuclear receptor subfamily 4, group A, member 2
1421818_at	4.039	Bcl6	B-cell leukemia/lymphoma 6
1452939_a_at	4.014	1110020B03Rik	RIKEN cDNA 1110020B03 gene
1454788_at	3.929	A630084M22Rik	RIKEN cDNA A630084M22 gene
1456064_at	3.887	Kcna3	mp78a12.x1 mRNA sequence.
1437725_x_at	3.799	2410018G23Rik	BB549567 RIKEN full-length mRNA sequence.
1434866_x_at	3.788	Cpt1a	BB021753 RIKEN full-length mRNA, mRNA sequence.
1417676_a_at	3.773	Ptpro	protein tyrosine phosphatase, receptor type, O
1454987_a_at	3.656	H2-Ke6	H2-K region expressed gene 6
1422751_at	3.653	Tle1	transducin-like enhancer of split 1, homolog of Drosophila E(spl)
1429722_at	3.619	9230111I22Rik	RIKEN cDNA 2310026P19 gene
1456333_a_at	3.535	Arhgap6	Rho GTPase activating protein 6
1416334_at	3.531	Wwox	WW domain-containing oxidoreductase
1460367_at	3.478	Hbp1	high mobility group box transcription factor 1
1430125_s_at	3.447	2310009N05Rik	RIKEN cDNA 2310009N05 gene
1443962_at	3.413	A330080J22Rik	RIKEN cDNA A330080J22 gene
1452948_at	3.394	1810019A08Rik	RIKEN cDNA 1810019A08 gene

1437114_at	3.371	A930001N09Rik	RIKEN cDNA A930001N09 gene
1450376_at	3.361	Mxi1	Max interacting protein 1
1426323_x_at	3.312	Siva	Cd27 binding protein (Hindu God of destruction)
1454967_at	3.258	A930001N09Rik	RIKEN cDNA A930001N09 gene
1452352_at	3.239	Ctla2b	trophoblast specific protein beta
1433680_x_at	3.239	Siva	Cd27 binding protein (Hindu God of destruction)
1449841_at	3.221	Kif3a	kinesin family member 3A
1454701_at	3.206	4930503L19Rik	RIKEN cDNA 4930503L19 gene
1442026_at	3.161	AI467657	Similar to mPLZF(B)=promyelocytic leukemia zinc finger protein mRNA
1436994_a_at	3.159	Hist1h1c	histone 1, H1c
1427194_a_at	3.14	Ckn1	Cockayne syndrome 1 homolog (human)
1451075_s_at	3.13	Ctdsp2	RIKEN full insert sequence
1419186_a_at	3.128	Siat8d	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D
1423411_at	3.105	BC013481	602884649F1 NCI_CGAP_Kid14 mRNA sequence.
1455078_at	3.094		Transcribed sequence
1451755_a_at	3.085	Apobec1	apolipoprotein B editing complex 1
1451715_at	3.074	Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B
1448575_at	3.065	Il7r	interleukin 7 receptor
1456378_s_at	3.047	2610511F20Rik	AV120094 mRNA sequence.
1448576_at	3.033	Il7r	interleukin 7 receptor
1431003_a_at	3.032	2610002J02Rik	RIKEN cDNA 2610002J02 gene
1436191_at	3.027	Arid4a	AT rich interactive domain 4A (Rbp1 like)
1434687_at	3.021	A930014K01Rik	RIKEN cDNA A930014K01 gene

Table 7.13 Genes uniquely downregulated by CyaA treatment for 6 h			
Gene Name	Fold Change	Name	Description
1422628_at	5.189	4632417K18Rik	expressed sequence AW413625
1418540_a_at	4.997	Ptpre	protein tyrosine phosphatase, receptor type, E
1422025_at	4.085	Mitf	microphthalmia-associated transcription factor
1417061_at	4.013	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1
1428900_s_at	3.936	0610027B03Rik	RIKEN cDNA 0610027B03 gene
1416988_at	3.917	Msh2	mutS homolog 2 (E. coli)
1428410_at	3.797	Mak3	Mak3p homolog (S. cerevisiae)
1439269_x_at	3.581	Mcm7	BB407228 RIKEN full-length enriched
1422440_at	3.522	Cdk4	cyclin-dependent kinase 4
1434740_at	3.342	Scarf2	scavenger receptor class F, member 2
1450871_a_at	3.326	Bcat1	branched chain aminotransferase 1, cytosolic
1456055_x_at	3.324	Pold1	polymerase (DNA directed), delta 1, catalytic subunit
1422147_a_at	3.257	Pla2g6	phospholipase A2, group VI
1416665_at	3.25	Coq7	demethyl-Q 7
1416309_at	3.174	Nusap1	nucleolar and spindle associated protein 1
1460247_a_at	3.106	Skp2	AV259620 RIKEN full-length mRNA sequence.
1455084_x_at	3.076	Shmt2	BB758291 RIKEN full-length enriched mRNA sequence.
1416951_a_at	3.075	Atp6v1d	ATPase, H+ transporting, V1 subunit D
1433581_at	3.073	1190002N15Rik	RIKEN cDNA 1190002N15 gene
1447943_x_at	3.066	BC042768	SMT3 (supressor of mif two, 3) homolog 2 (S. cerevisiae)
1425139_at	3.053	Sesn2	AV308638 RIKEN full-length mRNA sequence.
1452281_at	3.05	Sos2	Son of sevenless homolog 2 (Drosophila)
1428502_at	3.044	Actr6	ARP6 actin-related protein 6 homolog (yeast)
1434027_at	3.039	Dscr1l2	Down syndrome critical region gene 1-like 2
1419509_a_at	3.035	Nagk	N-acetylglucosamine kinase
1460456_at	3.03	2010316F05Rik	RIKEN cDNA A430106J12 gene
1436954_at	3.029	Waspip	Wiskott-Aldrich syndrome protein interacting protein
1437856_at	3.027	Impk	inositol polyphosphate multikinase
1428970_at	3.022	Mak3	Mak3p homolog (S. cerevisiae)
1435545_at	3.014	BC032203	BB242234 RIKEN full-length mRNA sequence.
1437325_x_at	3.006	Pycs	pyrroline-5-carboxylate synthetase

Table 7.14 Genes uniquely down regulated by co-treatment with CyaA and CpG at both 0.5 h and 6 h			
Gene Name	Fold Change	Name	Description
1423006_at	4.173	Pim1	proviral integration site 1
1435872_at	3.707	Pim1	proviral integration site 1
1419068_at	3.385	Rabgef1	RAB guanine nucleotide exchange factor (GEF) 1
1458299_s_at	3.092	Nfkbie	BB820441 RIKEN full-length enriched mRNA sequence.
1451466_at	3.089	D16Ertd472e	DNA segment, Chr 16, ERATO Doi 472, expressed
1430752_at	3.045	C330006D17Rik	BB397948 RIKEN mRNA sequence.
1435640_x_at	3.003		uv85h11.x1 Soares mouse 3NbMS mRNA sequence.

Table 7.15 Genes uniquely downregulated by co-treatment with CyaA and CpG at both 0.5 h and 6 h			
Gene Name	Fold Change	Common	Description
1428479_at	4.286	Nfatc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
1422535_at	3.84	Ccne2	cyclin E2
1443534_at	3.797		Transcribed sequences

Chapter 8

GENERAL DISCUSSION

8.1 General Discussion

An understanding of the mechanism of protective immunity against pathogenic microorganisms and the strategies employed by pathogens to evade immune responses is a critical process in the rational design of vaccines and treatments. The identification of the virulence factors that promote the survival and persistence of a pathogen has led to a greater knowledge of the interplay between pathogen and host and has helped to develop better sub-unit vaccines that stimulate protective immunity against infection.

Bordetella pertussis is a Gram-negative bacterium that causes a prolonged infection in humans and causes whooping cough (Mills, 2001). Infection with *B. pertussis* in immunocompetent individuals results in the generation of inflammatory immune responses that eventually clears the infection. However, infection in children or immunocompromised individuals can be prolonged and debilitating. Historically, the majority of *B. pertussis* cases have been reported in children under 10 years old (Cherry, 2003). The widespread introduction of an immunisation program using a whole cell pertussis vaccine, delivered as part of the tri-valent dipteria, tetnus, pertussis (DTP) vaccine, succeeded in reducing infections from a peak of over 250,000 cases per year in the United States in 1935 to less than 1000 cases per year in the latter half of the twentieth century. Concerns about reactogenicity of the whole cell pertussis vaccine in the 1990s lead to its replacement with an acellular pertussis vaccine composed of one to five components, including detoxified PT with or without FHA, pertactin and agglutinogens 2 and 3. However, there has been a steady rise in reported cases of pertussis in the last 20 years, even in countries where infants are routinely immunised (de Melker *et al.*, 2000). While not as numerous as the pre-vaccine era, there were almost 10,000 cases reported in the US in 2000 and more recent evidence suggests that this may be considerably higher. Alarmingly, adolescents and adults are those presenting with half of reported cases (Cherry, 2003).

This resurgence of pertussis has been attributed to several factors. Genetic variation of *B. pertussis* has been identified in clinical isolates. Although amino acid variation has been found in PT and pertactin, evidence to support the hypothesis that this may allow evasion of vaccine-induced immunity is still limited (de Melker *et al.*, 2000). Reduced potency of acellular pertussis vaccines is more of a concern. Many of

the acellular pertussis vaccines available have lower efficacy than whole cell pertussis vaccines and this may contribute to the resurgence of *B. pertussis* infection (Ntezayabo *et al.*, 2003). Waning immunity to *B. pertussis* may be the biggest cause of the increased incidence of disease. It appears that both vaccine and infection induced immunity against *B. pertussis* is not long lasting (Cherry, 1999). Adults and adolescents are becoming infected and developing disease, even if they have been immunised or previously been infected. A greater awareness of pertussis disease and better surveillance has also contributed to the increase in reported cases. Therefore *B. pertussis* remains an important pathogen and a better understanding of its pathogenesis may assist to eliminate it as a cause of disease. The development of modified DTP vaccines that are suitable for use in adults and adolescents and that prevent carriage of the infection may help to eliminate pertussis in humans (Cherry, 2003).

CyaA is an important virulence factor of *B. pertussis* and although considered to be a target of the protective immune response is not included in any of the current acellular pertussis vaccines (Cherry *et al.*, 2004). CyaA is secreted by *B. pertussis* and is required for virulence of the bacterium (Goodwin and Weiss, 1990). CyaA causes lysis and apoptosis in a variety of cells, including RBC, T cells and macrophages (Khelef *et al.*, 2001, Lee *et al.*, 1999, Westrop *et al.*, 1997). CyaA also inhibits important anti-bacterial functions such as chemotaxis, phagocytosis and superoxide generation (Friedman *et al.*, 1987, Njamkepo *et al.*, 2000, Pearson *et al.*, 1987). In addition, CyaA is an important danger signal utilised by the host defences to promote the clearance of the bacteria; it helps in recruitment of inflammatory cells into the lungs (Harvill *et al.*, 1999, Khelef *et al.*, 1994) and is a target for antibodies (Farfel *et al.*, 1990). Immunisation with CyaA protects against lethal challenge with *B. pertussis* (Guiso *et al.*, 1989) and also been shown to increase the antibody responses to the unrelated model antigen ovalbumin when co-injected in mice. (Hormozi *et al.*, 1999).

The work presented in this thesis explored how CyaA can regulate immune responses by modulating innate immune cells and thereby affecting the generation of antigen specific T cells. CyaA modulates the activation of macrophages and DC; this includes downregulation of the expression of the surface molecules CD40 and ICAM-I, which are associated with the generation of IFN-γ-producing Th1 cells. Furthermore,

CyaA suppresses TLR ligand-stimulated expression of these surface molecules and also suppresses TLR ligand-stimulated secretion of the pro-inflammatory cytokines, IL-12p70 and TNF-α and the chemokine MIP-1α. However, CyaA does have positive effects on activation of innate cells. CD80 expression was upregulated by CyaA and in synergy with TLR ligands IL-10 was secreted in high amounts. The data also demonstrated that this immunomodulation is dependent on binding of CyaA to the CD11b/CD18 integrin expressed on DC and macrophages. It had previously been reported that CD11b interacted with CyaA through the C-terminal domain of the protein (El-Azami-El-Idrissi *et al.*, 2003) and that CyaA could target DC *in vivo* through this interaction (Guermonprez *et al.*, 2002). FHA from *B. pertussis* also binds CD11b/CD18 on DC and stimulates IL-10 secretion (McGuirk *et al.*, 2002). CyaA has been shown to associate with FHA (Zaretzky *et al.*, 2002). The targeting of innate cells and suppression of their pro-inflammatory activities by *B. pertussis* virulence factors suggests a combined role for FHA and CyaA in suppressing the inflammatory response against *B. pertussis*.

CyaA is posttranslationally modified by the specific accessory protein CyaC, which acylates a palmitic fatty acid to Lys^{983}(Hackett *et al.*, 1994). It has been shown that NA-CyaA can still target and bind to cells expressing CD11b, but that acylation is required for the tight interaction of CyaA with the cell membrane and the formation of a pore (El-Azami-El-Idrissi *et al.*, 2003). Induction of cell lysis and death by CyaA has been attributed to this pore formation (El-Azami-El-Idrissi *et al.*, 2003, Gray *et al.*, 1998). The data presented here confirms these findings and demonstrated that NA-CyaA does not cause cell lysis and does not induce apoptosis of macrophages, while A-CyaA exerted these effects. The increased interaction of CyaA with the target cell membrane mediated by acylation of CyaA is thought to promote the oligomerisation of CyaA and the insertion of the N-terminal enzymatic domain across the eukaryotic cell membrane directly into the cytosol of the target cell (Gray *et al.*, 2001, Lee *et al.*, 2005b, Rogel and Hanski, 1992). However it has also been demonstrated in an artificial liposome system that acylation of CyaA is not required for it to penetrate the lipid bi-layer (Masin *et al.*, 2004). The data presented here clearly demonstrated that NA-CyaA retains the ability to modulate innate immune cells, suppressing inflammatory cytokine

production and promoting the secretion of the anti-inflammatory cytokine IL-10. However, it was also discovered that lowering the concentration of NA-CyaA reduced its modulatory effects on innate immune cells, while equivalent concentrations of A-CyaA retained their full modulatory abilities at the lower concentrations. This indicates that while acylation of CyaA is not essential for immunomodulatory effects, it does enhance it. Given that acylation of CyaA promotes tight binding to the target cell membrane and a change in protein conformation, it is possible that acylation enhances immunomodulation by CyaA by increasing its interactions with the target cell.

The N-terminal domain of CyaA contains an enzymatic domain that binds eukaryotic calmodulin upon entry to the target cell cytosol and becomes enzymatically active (Drum *et al.*, 2002, Gentile *et al.*, 1990, Sakamoto *et al.*, 1992). Once activated, the enzymatic domain is cleaved and is free in the cytosol (Rogel and Hanski, 1992), where it directly converts ATP to cAMP (Confer and Eaton, 1982). Acylation of CyaA was thought to be required for the translocation of the N-terminal domain into the cytosol of the cell and the subsequent accumulation of intracellular cAMP (El-Azami-El-Idrissi *et al.*, 2003, Gray *et al.*, 2001). In addition, cAMP induction by CyaA was shown to mediate modulation of surface marker expression on human DC, which could be mimicked by the artificial cAMP inducer, forskolin and inhibited by inhibitors of cAMP activated kinases and small molecule inhibitors of ATP binding by CyaA (Bagley *et al.*, 2002b, Soelaiman *et al.*, 2003). The data in this thesis clearly demonstrates that acylation of CyaA is not required for the accumulation of cAMP in the cytosol of host cells. While not necessary, acylation may augment the translocation of the enzymatic domain across the target cell membrane, thereby increasing the efficiency of the enzymatic reaction. The data presented here also demonstrates that iAC-CyaA, which cannot catalyse the formation of cAMP, can still bind to cells of the innate immune system and cause cell lysis and induce apoptosis. This is in agreement with a previous report demonstrating that CyaA lacking the enzymatic domain could still bind to innate cells (El-Azami-El-Idrissi *et al.*, 2003) and with observations that preventing the translocation of the enzymatic domain into the target cells does not prevent lysis (Gray *et al.*, 2001). In addition, iAC-CyaA does not synergise with TLR ligands to promote the secretion of IL-10, nor does it suppress TLR ligand-induced DC

co-stimulatory molecule expression or inflammatory cytokine secretion. This demonstrates that much of the modulatory effects of CyaA on innate immune cells are mediated through cAMP.

The demonstration that CyaA could enhance antibody responses to a co-injected antigen suggested that CyaA could modulate adaptive immune responses as well as innate immunity. CyaA targets DC *in vivo* through binding CD11b/CD18 (Guermonprez *et al.*, 2002). CyaA molecules constructed with T cell epitopes from other antigens within the N-terminal domain have been shown to be an effective means for delivery of these epitopes to the cytosol where they are processed and presented by MHC class-I molecules to $CD8^+$ T cells (Simsova *et al.*, 2004). Immunisation with a CyaA molecule carrying a viral or tumour antigen has been shown to elicit immunity against viral and tumour challenge *in vivo*. Furthermore, the induction of *M. tuberculosis* specific T cells was also induced by this method of antigen delivery by CyaA (Fayolle *et al.*, 1999, Saron *et al.*, 1997, Wilkinson *et al.*, 2005). CyaA was also shown to deliver a T cell epitope to the MHC class-II pathway for presentation to $CD4^+$ T cells (Loucka *et al.*, 2002). Delivery of *M. tuberculosis*, HIV and LCMV epitopes by CyaA molecules have generated both $CD4^+$ and $CD8^+$ epitope-specific T cells (Dadaglio *et al.*, 2003, Mascarell *et al.*, 2005, Wilkinson *et al.*, 2005). These $CD4^+$ T cells were characterised and classified as IFN-γ producing Th1 cells. The generation of the CD4+ T cell response is dependant on binding to CD11b and also antigen presentation by MHC class-II molecules (Schlecht *et al.*, 2004, Wilkinson *et al.*, 2005). In addition, the enzymatic domain does not need to be active for efficient antigen presentation and activation of epitope specific T cell responses (Dadaglio *et al.*, 2003, Fayolle *et al.*, 1999, Fayolle *et al.*, 2001, Loucka *et al.*, 2002, Mascarell *et al.*, 2005, Sebo *et al.*, 1999, Wilkinson *et al.*, 2005). This indicates that the induction of T cell responses specific for foreign epitopes delivered by CyaA molecules is mediated through a mechanism other than cAMP induction in the target cell.

The results presented in this thesis demonstrate for the first time that CyaA induces $CD4^+$ Th2 and Tr1 cells specific for a co-injected antigen. In addition, antigen-specific T cells from mice immunised with antigen and CyaA were cloned and their cytokine profiles demonstrated the induction of Th2 clones, which secreted IL-4, IL-10,

IL-5, and Tr1 clones that secreted IL-10 or IL-10 and IL-5 but no IFN-γ. CyaA also promoted the production of antigen-specific IgG antibodies and these were polarised to the IgG1 subclass, which is consistent with the induction of Th2 cells but not Th1 cells. CyaA also generated these responses against an antigen that was delivered with CyaA by the nasal route, where it also promoted the production of antigen-specific IgA in the lungs. These results demonstrated that CyaA can generate both local, mucosal as well as systemic immunity. The acylation status of the molecule did not affect the type or magnitude of the adaptive response induced by CyaA. Antigen-specific responses generated by both A-CyaA and NA-CyaA were virtually indistinguishable, with both promoting the generation of Th2 cells, IL-10 secreting Tr1 cells and IgG1 antibodies. However, the enzymatic activity of CyaA did play a significant role in the generation of adaptive immune responses. KLH-specific cytokine production was not detected in mice immunised with KLH in the presence of iAC-CyaA. In addition, antigen-specific antibody production in these mice was not statistically different from that in mice immunised with antigen alone. This indicates that the induction of cAMP in innate immune cells may be an important function of CyaA in the generation of adaptive immunity. However, it was observed that there was a small, but statistically significant increase in antigen-specific antibody production induced by co-injection of antigen with iAC-CyaA; this was evident follow a booster immunisation. However this antibody response was significantly lower than that generated by immunisation with antigen in the presence of A-CyaA or NA-CyaA. This indicates that in addition to cAMP induction, CyaA may also mediate its adjuvant effects through alternative mechanisms that may arise from binding to the cell surface receptor CD11b/CD18 on DC.

These results provide a potential mechanism for the modulation of the immune response by CyaA. CyaA targets and modulates cells of the innate immune system expressing CD11b/CD18 and in synergy with TLR-agonists induces the secretion of the anti-inflammatory cytokine, IL-10 and suppresses production of inflammatory cytokines. In addition, certain DC surface markers required for Th1 cell co-stimulation are downregulated. cAMP, but not acylation of CyaA, is required for this modulation of innate immune cells. Acylation of CyaA improves the interaction with the target cell

and increases the efficiency of the response. The characteristics of these modulated innate immune cells are similar to those that have been previously been shown to promote the induction of Tr1 cells. The CyaA modulated DC appear to selectively promote Tr1 and Th2 cell induction, possible through the induction of innate IL-10 secretion, but also upregulation of CD80 expression and suppression of IL-12p70 secretion and CD40 and ICAM-I expression.

The induction of Tr1 cells by CyaA is interesting given that the response to *B. pertussis* is mediated by IFN-γ producing Th1 and NK cells (Byrne *et al.*, 2004, Mahon *et al.*, 1997, Mills *et al.*, 1998). The role of IL-10 secreting Tr1 cells in infection is only beginning to be understood. Regulatory T cells control immune responses to bacteria, viruses and fungi by regulating the inflammatory response.

Pro-inflammatory responses are necessary to control bacterial infections and infections with other pathogens. Inflammatory responses can be characterised by the secretion of pro-inflammatory cytokines and chemokines from innate immune cells and the recruitment of pathogen-specific inflammatory cells to the site of infection. These responses are typically accompanied by the activation of cytotoxic cells that lyse infected or damaged cells. Immunological memory is also generated to protect against subsequent infections. While the inflammatory response is necessary to eliminate the infection it can result in severe inflammation and damage to host tissues if left uncontrolled. The induction of anti-inflammatory cytokines in response to pathogen derived products is one mechanism by which the pro-inflammatory responses are suppressed. Secretion of IL-10 and the inhibition of IL-12p70 and TNF-α secretion from DC and macrophages in response to CyaA likely represents a mechanism through which the host immune response recognises CyaA as a marker of *B. pertussis* infection and initiate a response to regulate the anti-bacterial inflammation.

It is becoming clear that the adaptive immune response also plays a role in regulating the inflammatory responses against pathogens through the development of Th2 and regulatory T cells. Th2 cells secrete IL-10 and IL-4 that can regulate inflammation. Regulatory T cells produce IL-10 and are also induced in response to infection and limit immune mediated pathology caused by the anti-pathogen inflammatory response. Pathogen-specific regulatory T cells have been found during

infection with HCV (MacDonald *et al.*, 2002), Epstein-Barr virus (Marshall *et al.*, 2003), *M. tuberculosis* (Boussiotis *et al.*, 2000) and HIV (Ostrowski *et al.*, 2001). It has been shown that Tr1 cells are induced in the lung during *B. pertussis* infection where they suppress Th1 responses but also limit inflammation during the clearance of the infection (Higgins *et al.*, 2003, McGuirk *et al.*, 2002).

IL-10 is a differentiation factor for Tr1 cells *in vivo* (McGuirk *et al.*, 2002). Tr1 cells can be generated *in vitro* by repeated stimulation with IL-10 in the presence of the appropriate antigen and can suppress Th1 responses (Groux *et al.*, 1997). DC promote the differentiation of T cells into effector subtypes through the secretion of cytokines, the expression of surface co-stimulatory molecules and the presentation of antigen. There is evidence that DC with a particular activation status may promote the generation of Tr1 cells (Jonuleit *et al.*, 2000) and that these DC have an intermediate phenotype that can be characterised as having high MHC class-II expression but low surface expression of CD40 and ICAM-I (Caramalho *et al.*, 2003, Lavelle *et al.*, 2003). These DC also secrete high levels of IL-10 but low levels of IL-12. It is possible that the presence of high amounts of IL-10 and the lack of IL-12p70 in the cytokine milieu together with antigen stimulation but in the absence of pro-inflammatory co-stimulation from CD40 and ICAM-I promotes the generation of IL-10 secreting Tr1 cells. Indeed, pathogen-derived molecules, including CT and FHA, can promote the generation of this DC phenotype alone or in synergy with TLR ligands (Lavelle *et al.*, 2003, McGuirk *et al.*, 2002). The data in this thesis has shown that CyaA promotes a similar DC phenotype characterised by the secretion of high levels of IL-10 in the absence of inflammatory cytokines and the expression of MHC class-II without an upregulation of surface expressed CD40 or ICAM-I. It is likely that this DC phenotype activated by CyaA promotes the generation of Tr1 cells.

The mechanism of suppression by Tr1 cells is thought to be through the secretion of the immunosuppressive cytokine IL-10 rather than through cell to cell contact. Blocking IL-10 with antibodies inhibits the regulatory effects of Tr1 cells and Tr1 cells can also regulate Th1 responses *in vitro* across a semi-permeable membrane (Lavelle *et al.*, 2003, McGuirk *et al.*, 2002). Tr1 cells can also suppress the activity of pathogen-specific Th1 cells *in vivo* (McGuirk *et al.*, 2002). Indeed, the suppression of

inflammatory responses might be enhanced by innate immune cells by their production of IL-10 in response to pathogen-derived molecules. Indeed, IL-10 produced from innate cells does facilitate the induction of Tr1 cells (McGuirk *et al.*, 2002). Further studies have shown that IL-10 produced by DC and by macrophages promotes the generation of Tr1 cells during *B. pertussis* infection (Higgins *et al.*, 2003). IL-10 produced by both innate cells and Tr1 cells may help to limit immune mediated pathology caused during the infection. In the absence of this regulatory IL-10 the bacterial load is increased during *B. pertussis* infection and lung damage is more severe (Higgins *et al.*, 2003). It is possible that regulatory T cells are induced during infection with most, if not all pathogens as a method to suppress the damaging inflammatory responses and to suppress immune responses once the pathogen has been eliminated.

While regulatory T cells are of benefit to the host it is also possible that pathogens can exploit the induction of regulatory T cells to subvert protective immune responses facilitating persistence and the establishment of chronic infection. IL-10 secreting Tr1 cells are produced during chronic and persistent infections with virus, bacteria and parasite infections, including HCV, HIV, EBV, Friend virus, *M. tuberculosis, B. pertussis* and *Leismania major* (Boussiotis *et al.*, 2000, Dittmer *et al.*, 2004, Marshall *et al.*, 2003, McGuirk *et al.*, 2002, Ostrowski *et al.*, 2001, Sacks and Noben-Trauth, 2002). These Tr1 cells play a role in suppressing the inflammatory response against the pathogen, which can be blocked using antibodies against IL-10. The induction of Tr1 cells by pathogens may represent a pathway evolved by the pathogen to suppress the Th1 response normally induced to eliminate the pathogen. In addition to the induction of Tr1 cells during *B. pertussis* infection (Higgins *et al.*, 2003), FHA from *B. pertussis* also promotes the generation of Tr1 cells through the modulation of DC and the induction of IL-10 secretion (McGuirk *et al.*, 2002). The promotion of IL-10 secretion from DC together with the suppression of pro-inflammatory cytokines by CyaA indicates a further mechanism for the induction of antigen-specific IL-10 secreting Tr1 cells by *B. pertussis*. This modulation of the immune system by CyaA likely plays a role in the suppression of inflammatory responses against *B. pertussis* during infection.

Tr1 cells play an important role in the maintenance of homeostasis in the immune system. Autoimmune diseases can occur from an inability to control inflammation and the development of an immune response against self and in some cases has been linked to defects in regulatory T cells (Banchereau and Steinman, 1998, Mills, 2004). The induction or transfer of regulatory T cells has been shown to ameliorate the symptoms of some immune-mediated disorders (Chen *et al.*, 1994). It may be possible therefore to induce Tr1 cells that suppress the inflammatory mediators in autoimmune diseases using molecules derived from pathogens. CyaA has potential to induce Tr1 cells specific for self-antigens *in vivo* as a therapy for autoimmune diseases. However, the use of pathogen derived molecules as therapies for human conditions is not straightforward with many regulatory requirements. One of these is requirements is a lack toxicity, wildtype A-CyaA is a potent immunomodulatory but does cause cause cell death. However, NA-CyaA retains immunomodulatory activity and is non-toxic. Therefore NA-CyaA has considerable potential as a therapeutic agent for immune-mediated conditions.

It is clear that CyaA is a powerful immunomodulatory molecule that regulates the activation of innate immune cells and promotes the generation of regulatory T cells. These responses can be both harmful and beneficial to the host by suppressing anti-bacterial functions and regulating immune mediated pathology during the clearance of infection. The results of this study have provided insights into the importance of the structure of CyaA for regulating immune cell function. In addition, a study of the modulation of gene transcription in innate cells has revealed that CyaA suppresses inflammatory genes and promotes the upregulation genes associated with the induction of a regulatory immune phenotype and also upregulates genes that suppress inflammation. The understanding of how CyaA modulates the immune system has provided an insight into how *B. pertussis* modulates the immune system during infection and how the host responds to infection. In addition, these results have added to the understanding of how the immune system is activated, and responds to infection and may help to identify therapeutic targets and treatments for immune-mediated conditions.

CHAPTER 9

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APPENDIX I

PLASMID SEQUENCES

Appendix I Plasmid sequences

CyaA gene sequences are indicated in blue.
CyaC gene sequences are indicated in red.
6xHIS-tag highlighted in red
Start codon highlighted in green
Stop codon highlighted in yellow
Mutated bases are indicated in green italics and underlined.

I.1 pJR2

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   1 CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
  51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
 101 AGGAGAAATT AACTATGAGA GGATCGCATC ACCATCACCA TCACGGATCC
 151 GCATGCGAGC TCGGTACCAT GCAGCAATCG CATCAGGCTG GTTACGCAAA
 201 CGCCGCCGAC CGGGAGTCTG GCATCCCCGC AGCCGTACTC GATGGCATCA
 251 AGGCCGTGGC GAAGGAAAAA AACGCCACAT TGATGTTCCG CCTGGTCAAC
 301 CCCCATTCCA CCAGCCTGAT TGCCGAAGGG GTGGCCACCA AAGGATTGGG
 351 CGTGCACGCC AAGTCGTCCG ATTGGGGGTT GCAGGCGGGC TACATTCCCG
 401 TCAACCCGAA TCTTTCCAAA CTGTTCGGCC GTGCGCCCGA GGTGATCGCG
 451 CGGGCCGACA ACGACGTCAA CAGCAGCCTG GCGCATGGCC ATACCGCGGT
 501 CGACCTGACG CTGTCGAAAG AGCGGCTTGA CTATCTGCGG CAAGCGGGCC
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 601 GAGCAGTTCG AGTTTCGCGT GAAGGAAACC TCGGACGGGC GCTATGCCGT
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 851 AGGCCACGGG CGGCCTGGAT CGCGAACGCA TCGACTTGTT GTGGAAAATC
 901 GCTCGCGCCG GCGCCCGTTC CGCAGTGGGC ACCGAGGCGC GTCGCCAGTT
 951 CCGCTACGAC GGCGACATGA ATATCGGCGT GATCACCGAT TTCGAGCTGG
1001 AAGTGCGCAA TGCGCTGAAC AGGCGGGCGC ACGCCGTCGG CGCGCAGGAC
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1101 GAAGATTTTC GTCGTATCGG CCACCGGTGA AAGCCAGATG CTCACGCGCG
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1451 CGGCGGAACT GGAAATGACC CGGCAAGTCT TGCACGCCGG GGCGCGGCAG
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