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Mechanisms of Immunomodulatory Activity of Cholera Toxin

Thesis submitted to the University of Dublin For the Degree of Doctor of Philosophy

By

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Department of Biochemistry
Trinity College
Dublin Ireland
January 2005
Declaration

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Olive Leavy
Abstract

Cholera toxin (CT), a major enterotoxin produced by *Vibrio cholerae*, is a potent mucosal adjuvant that promotes type 2 T helper cell (Th2) responses to coadministered foreign antigen. However, the mechanisms involved in the regulation of immune responses by CT are not known. This study examined the ability of CT to modulate the maturation and activation of bone marrow derived dendritic cells (DCs). It also addressed the hypothesis that the adjuvant activity of CT reflects its ability to activate T cells as well as DCs.

CT induced the maturation of DCs, as demonstrated through the upregulation of CD80 and CD86 expression and induction of macrophage inflammatory protein-2 (MIP-2) production. CT also synergised with low doses of lipopolysaccharide (LPS) to enhance IL-10 production but inhibited IL-12 production from immature DCs. An investigation of the involvement of the mitogen-activated protein (MAP) kinases in the modulatory activity of CT in DCs demonstrated that CT induced the prolonged phosphorylation of extracellular signal regulated kinase (ERK) MAP kinase but not p38. In addition, CT also activated the immediate early transcription factor c-fos in DCs. U0126, a specific inhibitor for ERK, inhibited the production of MIP-2 and partially reversed the upregulation of CD80 in response to CT. ERK phosphorylation also contributed to the enhancement of LPS-induced IL-10 and inhibition of IL-12 by CT, demonstrating an important role for this kinase in the mechanism of action of CT in DCs.

In addition to its effects on DCs, CT directly modulated cytokine production from T cells, enhancing Th2, but suppressing Th1-associated cytokines. CT inhibited cytokine production by CD3-activated naïve T cells and IFN-γ production by Th1 clones, but enhanced IL-4, IL-5 and IL-10 production by mixed Th1/Th2 cell lines, IL-5 production by type 1 regulatory T (Tr1) clones and directly induced IL-5 production from the T cell line EL4.IL-2. CT promoted the phosphorylation of both ERK and p38 in EL4.IL-2 cells and inhibition of these kinases, using U0126 or SB203580 respectively, demonstrated an essential role of both of these kinases in CT-induced IL-5 production by EL4.IL-2 cells.

Therefore, the modulation of immune responses by CT is a multi-factorial process, involving direct interaction with T cells as well as DCs and involves the ERK and p38 MAP kinases and the transcription factor, c-fos.
Acknowledgements

First and foremost I would like to thank a fellow Westmeath scientist, my supervisor Professor Kingston Mills. Without your support and encouragement, especially during the more difficult periods of the last four years, I really don’t believe this project would ever have been completed. A very special thank you must also go to Ed and Andy. Your constant source of encouragement, faith in my ability, ideas but most of all your friendship has played a role bigger than you will ever know in this project.

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This work is dedicated to you both
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<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>AP</td>
<td>Activator Protein</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
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<tr>
<td>ARF</td>
<td>ADP-ribosylation factors</td>
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<td>ATF</td>
<td>Activating Transcription Factor</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BSA</td>
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<td>CaMk</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
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<td>cAMP</td>
<td>Cyclic 3',5'-adenosine monophosphate</td>
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<td>CBP</td>
<td>cAMP binding protein</td>
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<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
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<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<td>c-IAP2</td>
<td>cellular inhibitor of the apoptosis protein 2</td>
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<td>c-Maf</td>
<td>c-musculoaponeurotic fibrosarcoma</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>I kB</td>
<td>Inhibitor of NFkB</td>
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<tr>
<td>IKK</td>
<td>I kB Kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-1R</td>
<td>IL-1 receptor</td>
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<td>IL-1Ra</td>
<td>IL-1R antagonist</td>
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<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
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<tr>
<td>IP</td>
<td>IFN-γ Inducible Protein</td>
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<tr>
<td>IRAK</td>
<td>IL-1R-Associated Kinase</td>
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<tr>
<td>IRE</td>
<td>IFN-γ-response element</td>
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<td>IRF</td>
<td>IFN regulator factor</td>
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<td>ISCOMS</td>
<td>Immune-Stimulating Complexes</td>
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<td>ISRE</td>
<td>IFN Stimulated Response Element</td>
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<tr>
<td>JAK</td>
<td>Janus-family Tyrosine Kinase</td>
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<tr>
<td>JNK</td>
<td>c-jun NH$_2$-Terminal Kinase</td>
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KLH  Keyhole Limpet Hemocyanin
LAL  Limulus Amebocyte Lysate
LBP  LPS-Binding Protein
LFA  Lymphocyte Function-Associated Antigen
LFH  Liver Fluke Homogenate
LN   Lymph Node
LPS  Lipopolysaccharide
LT   Heat-Labile Enterotoxin
Mal  MyD88-Adaptor like Protein
MAP  Mitogen Activated Protein
MBP  Myelin basic protein
MCP  Macrophage Chemoattractant Protein
MEK  MAP Kinase/ERK Kinase
MFI  Mean Fluorescence Intensity
MHC  Major Histocompatibility Complex
MIP  Macrophage Inflammatory Protein
MKK  MAP Kinase Kinase
MKKK MAP Kinase Kinase Kinase
MPL  Monophosphoryl Lipid A
mRNA Messenger RNA
MSK  Mitogen- and Stress-activated Protein Kinase
MyD88 Myeloid Differentiation Factor 88
NEMO NFκB Essential Modulator
NFAT Nuclear Factor of Activation in T cells
NFκB Nuclear Factor κB
ng   Nanogram
NK   Natural Killer
NOD  Nonobese Diabetic
OD   Optical Density
OPD  o-phenylenediamine
OVA  Ovalbumin
PAMP Pathogen Associated Molecular Pattern
PBMC Peripheral Blood Mononuclear Cell
PBS Phosphate Buffer Saline
PE Phycoerythrin
pg picogram
PGE$_2$ Prostaglandin E$_2$
PKA/C Protein Kinase A/C
PLG poly (D,L-lactide-co-glycolide) microparticles
PMA phorpol myristic acetate
PRR Pattern Recognition Receptor
PT Pertussis Toxin
RANTES Regulated on Activation Normal T cell Expressed and Secreted
rIL-2 recombinant IL-2
RNA ribonucleic acid
RPMI Roswell Park Memorial Institute medium
RT Room Temperature
RT-PCR Reverse Transcriptase-Polymerase Chain Reaction
s.c. subcutaneous
SD Standard Deviation
SDS Sodium Dodecylsulphate
SEA schistosome egg antigens
SOCS Suppressor of Cytokine Signalling
STAT Signal transducer and activator of transcription
TAB TAK-1 Binding Protein
TAK Transforming Growth Factor-β-Activated Kinase
T-bet T-box Expressed in T cells
TCR T Cell Receptor
TEMED N, N, N', N'-Tetramethylethylenediamine
TGF Transforming Growth Factor
Th T Helper
TIR Toll/IL-1R resistance
TIRAP TIR Domain-Containing Adaptor Protein
TLR Toll Like Receptor
TNF Tumor Necrosis Factor
Tr T regulatory
TRAF TNFR-Associated Factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR domain-containing adapter-inducing IFN-β</td>
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<td>UV</td>
<td>Ultra Violet</td>
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Chapter One

General Introduction
1.1 General Introduction

The development of vaccines has had a significant impact on human health since their introduction, with the prevention of many millions of deaths each year from infectious diseases. Most modern vaccines are now produced using purified native recombinant proteins or protein conjugate polysaccharides, and are generally safer than traditional vaccines, which are based on attenuated or inactivated bacteria or viruses. However, these purified or recombinant vaccines are often poorly immunogenic. This is mainly due to the fact that the protein or polysaccharide antigens are more refined than traditional vaccines and, therefore, do not contain extraneous bacterial or viral components, which often function as built-in adjuvants. Because of this, adjuvants are often required to enhance the immunogenicity of the purified or recombinant proteins.

Cholera toxin (CT) is a potent adjuvant, enhancing the immune response to coadministered antigens. To understand how this toxin enhances the immune response we first must understand its effect on cells of the immune system and the signalling pathways activated in these cells. Furthermore, CT can be used as a powerful tool to decipher the mechanisms involved in the polarisation of immune responses following encounter with bacterial proteins.

1.2 Immunity to Infection

Protective immunity results from the interplay of two cardinal systems: the innate immune system and the acquired or adaptive immune system. The innate immune system is the first line of immunological defence against infection, consisting of cells and molecules that can immediately respond to a wide range of pathogenic microbes without requiring prior exposure. In contrast, the adaptive immune system is
mediated by lymphocytes, which are under the control of cells of the innate immune system. Studies in human and animal models have revealed that the ability of a host to effectively eradicate an invading organism depends on the generation of the appropriate type of cell-mediated and/or humoral immune response (discussed below). Some lymphocytes may also develop into memory cells that have the capacity to mount a rapid and effective immune response upon subsequent exposure to an antigen (Ag). The type of immune response induced plays a critical role in the outcome of an infection in terms of both protective immunity and immunopathology.

1.2.1 The Innate Immune Response

The innate immune system is mediated largely by white blood cells, such as neutrophils, macrophages and dendritic cells; cells that phagocytose and kill invading pathogens. The innate immune system also controls many aspects of adaptive immunity through the recognition of infectious microbes and as a consequence, the induction of signals that direct the induction of distinct types of responses (Medzhitov and Janeway, 1997). Certain cells of the innate immune system recognise conserved structures, known as pathogen associated molecular patterns (PAMPs), on the microbe through pattern recognition receptors (PRRs) on their surface. These receptors include Toll-like receptors (TLRs), CD14, complement receptors, lectins (e.g. mannose receptor, DEC-205 and DC-SIGN), Fc receptors and CD11b,c/CD18 (Fearon and Locksley, 1996; Palucka and Banchereau, 2002). PRRs are strategically placed on cells that are first to encounter invading pathogens, such as epithelial cells and APCs, and allow distinction of the infectious agent from self and discrimination among pathogens (Medzhitov and Janeway, 1997). The innate immune system has
the important role of recognising invading pathogens and initiates and controls the appropriate adaptive immune response that is best suited to defend the host.

1.2.2 Adaptive Immunity

The adaptive immune system consists of two main classes of lymphocytes: B cells (B lymphocytes), which are responsible for humoral immunity and the production of antibodies, and T cells (T lymphocytes), which function mainly in cell-mediated immune responses. CD8\(^+\) cytotoxic T lymphocytes (CTL) defend against intracellular pathogens by binding to and lysing the infected cells. CD4\(^+\) T helper (Th) cells produce cytokines, which mobilize both humoral and cell-mediated branches of the immune response, as well as activating cells of the innate immune system. The adaptive immune response is initiated when CD4\(^+\) Th cells recognize foreign peptide bound to the major histocompatibility complex (MHC) class II expressed on certain cells of the innate immune system know as antigen presenting cells (APCs). According to current dogma, CD4\(^+\) Th cells can differentiate towards at least two subtypes of Th cells, namely Th1 and Th2. These subsets are identified mainly by their cytokine profile and possess distinct functions in immunity to infection. Murine Th1 cells produce interleukin (IL)-2 and interferon (IFN)-\(\gamma\) and promote cellular immunity, such as activation of cytotoxic and phagocytic functions in effector cells and delayed-type hypersensitivity (DTH). Th2 cells, which secrete IL-4, IL-5, IL-10 and IL-13, regulate B cell production of antibodies, and provide protection from helminth infection but can also mediate pathological conditions such as asthma and allergy (Mosmann and Coffman, 1989). More recently, a number of CD4\(^+\) regulatory T cell (Tr) subsets have been identified. These Tr populations can be loosely divided into three groups, based on their mode of suppression. Natural
CD4^CD25^ Tr cells exert their suppressive effects through a contact dependent mechanism. In contrast, inducible type 1 Tr cells (Tr1) and type 3 Th cells (Th3) suppress responses via a contact independent/cytokine dependent mechanism that is based mainly on the production of IL-10 and/or transforming growth factor (TGF)-β (McGuirk and Mills, 2002). However, in many cases the immune response is not polarised toward a single distinct subtype and therefore the balance between these T cell subsets often determines whether an invading pathogen persists or is eradicated (Fig. 1.1.).

Many studies have illustrated the essential role of Th subsets in disease conditions, where the development of one Th subset is associated with susceptibility to the disease, whereas expansion of an opposing subset results in disease resistance. For example, infection with *Mycobacterium leprae* can result in different clinical forms of leprosy depending on the Th response induced (Modlin, 1994). In patients with tuberculoid leprosy, in which Th1 cells are preferentially induced and efficient macrophage activation occurs, the disease progresses slowly and the patient usually survives. In lepromatous leprosy, however, Th2-associated cytokines are dominant and cell-mediated immunity is profoundly suppressed, which results in anergy and disseminated infection. This form of leprosy is generally fatal. Therefore, understanding the mechanisms involved in the generation of polarised Th cell populations is essential in the generation of effective vaccines and therapies against infectious pathogens.

### 1.3 Dendritic cells (DCs)

DCs play a pivotal role in the initiation of adaptive immunity in the host. In their immature form they act as sentinels for ‘danger’ signals in the peripheral tissues,
Figure 1.1 Schematic representation of the dichotomy of Th cell differentiation
Activation of naïve CD4⁺ Th0 cells results in the production of IL-2, which stimulates the proliferation of Th cells, and their differentiation into Th1, Th2 or Tr1 effector cells. The cytokine milieu at the time of activation is the dominant regulator in this differentiation. Th1 cells promote cell-mediated immunity, Th2 cell are associated with the development of humoral immunity, while Tr1 cells have suppressor/regulator functions.
where they encounter and respond to invading pathogens or foreign Ag. Following maturation, DCs migrate to the lymph nodes (LN) where they activate and control the differentiation of naïve T cells, thus initiating primary immune responses (Banchereau and Steinman, 1998). Although all DCs share phenotypic and functional properties, two distinct subsets, based on the origin of the cell (myeloid or lymphoid) have been identified. In mouse, these lineages can be identified by the differential expression of certain surface markers. DCs derived from a lymphoid origin are thought to be CD11c⁺, CD11b<sup>dull</sup>, with high levels of CD8α and DEC-205 lectin (so-called ‘CD8α⁺ lymphoid’ DCs) (Wu et al., 1995). Another group of DCs are found within the CD11c⁺, CD11b<sup>bright</sup> subset and do not express CD8α and DEC-205 lectin. However, they do express CD4⁺ and the myeloid-related markers F4-80 and 33D-1, suggesting their myeloid origin (so-called ‘CD8α⁺ CD4⁺ myeloid’ DCs) (Pulendran et al., 1997). These myeloid cells can be generated from mouse bone marrow or blood upon culture with granulocyte/macrophage-colony stimulating factor (GM-CSF) (Inaba et al., 1992).

These subsets of DCs may vary in their microenvironmental localization and functional specialization. CD8α⁺ DCs have been identified in the lymphoid organs, spleen and Peyer’s patches, specifically in the T cell areas. It has been suggested that this subset produce high concentrations of Th1 polarising cytokine, IL-12p70, in response to a variety of stimuli and promote Th1-associated immune responses. In contrast, the CD8α⁻ DC subset is primarily located in the non-lymphoid organs or at sites where cells and Ags first enter the lymph organs. Myeloid DCs may not produce much IL-12p70 and promote the induction of a Th2-associated T cell response (MacDonald et al., 2001; Maldonado-Lopez et al., 1999; Pulendran et al., 1997). Also, CD8α⁻ myeloid DCs produce high levels of IL-10 when stimulated with the
TLR2-ligands zymosan (yeast derivative) or the synthetic ligand Pam-3-cys (Dillon et al., 2004; Edwards et al., 2002). However, there is also strong evidence that these subsets are more flexible and are not rigidly confined to a Th1- or Th2-inducing capacity based on lineage, and instead can respond to the nature of the microbial stimulus, the local environment (i.e. cytokine environment) and the dose of Ag (Maldonado-Lopez et al., 2001; McGuirk and Mills, 2002).

1.3.1 Maturation of DCs

In the periphery, DCs are found in an immature state, prior to encounter with pathogenic molecules and inflammatory mediators. In this state, DCs are very efficient at capturing microbes, utilizing several mechanisms such as macropinocytosis, receptor-mediated endocytosis and phagocytosis. Expression of PRRs, such as TLRs on the surface of DCs, facilitates the DC to respond to conserved microbial molecules or PAMPs. Immature DCs express low levels of the costimulatory molecules CD80 and CD86, MHC class II and other costimulatory molecules such as CD40 and OX40 ligand (OX40L) and therefore do not effectively present Ag to T cells. Upon interaction with microbes or microbial products, DCs begin the maturation process and migrate to lymphoid tissues, such as the LN and spleen. This migration is governed by the coordinated action of several chemokines. Certain chemokine receptors, such as CCR5 and CCR6, which are involved in the recruitment of DCs to the site of inflammation, are downregulated and the DC becomes less responsive to chemokines specific for immature DCs (e.g. macrophage inflammatory protein-3α (MIP-3α)). Other chemokine receptors are upregulated, for example CCR7, a receptor for the chemokine MIP-3β, which is expressed in the T-cell area of LN, thereby facilitating the movement of the DC towards the lymphoid
tissue. The maturing DCs can also produce certain chemokines, which attract other DCs and lymphocytes to the sight of infection. On route to the LN, the DCs undergo a maturation process, characterised by the loss of phagocytic/endocytic receptors, the upregulation of MHC class II, co-stimulatory molecules, activation of signalling pathways and an increase in cytokine production. (Banchereau et al., 2000; Moser and Murphy, 2000; Sato et al., 2001).

Following encounter with pathogens or PAMPs in the tissue and upon arrival in the draining LN, the DC is a fully functional APC and processes the Ag for presentation in association with MHC class II and provides the necessary signals for activation of naïve T cells. These signals are provided through engagement of the T cell receptor (TCR) with the MHC/peptide complex and through the engagement of the co-stimulatory molecules on the DC with their specific ligands on the T cell.

It has recently been proposed that the DC also provides an additional signal that contributes to the initial commitment of naïve T cells towards polarised effector Th cells. Depending on the maturation stimulus and the environmental signals received by the DC upon activation, it is proposed that at least three functional subsets of DC may be generated with the ability to induce different Th cell responses: 1) a subset with high costimulatory capacity and high IL-12 production (DC1), 2) a subset with high costimulatory capacity and low IL-12 production (DC2) and 3) a subset with low costimulatory capacity, low IL-12 production and high IL-10 (DCr) (Kalinski et al., 1999; McGuirk et al., 2002). DC1 promotes Th1 type responses whereas DC2 promote Th2 polarisation and DCr direct naïve T cells towards a Tr1 phenotype (Fig. 1.2).

Therefore, effective polarisation of Th cells is dependent on a specific signal provided by the DC, through the release of particular cytokines into the surrounding
Figure 1.2 Proposed model for T helper (Th) cell differentiation in response to pathogenic molecules through dendritic cell (DC) modulation

Certain pathogenic molecules bind to and stimulate the activation of immature DCs (iDCs) into DC1 and DC2, which promote the direct differentiation of Th1 (IFN-$\gamma$-dominant) or Th2 (IL-4-dominant) cells respectively. Other molecules direct iDCs to promote the development of regulatory T cells (Tr) (termed DCr). These Tr cells can suppress Th1 and Th2 cell development through contact-dependent or anti-inflammatory cytokine-dependent mechanisms. Abbreviations: LPS, lipopolysaccharide; FHA, filamentous haemagglutinin, CT, cholera toxin; IFN, interferon; LT, labile enterotoxin; TGF, transforming growth factor.

Reproduced from McGuirk and Mills, 2002
environment and/or through a direct signal to the T cell. Microbial products, such as lipopolysaccharide (LPS), CpG, bacterial toxins, pathogen-derived molecules such as filamentous haemaglutinin (FHA), allergens, helminths and double-stranded RNA (dsRNA) have the capacity to activate/modulate costimulatory molecule expression and cytokine production by DCs and are therefore important stimuli required by DCs for directing T cell responses.

1.3.2 Toll-like receptors (TLRs)

The TLRs are an evolutionarily conserved family of PRRs, part of the IL-1 receptor (IL-1R)/TLR superfamily, that are important in the recognition of microbes or microbial products (Akira et al., 2001; Janeway and Medzhitov, 2002). To date, thirteen TLRs have been identified in and on a variety of murine immune cells. The TLR family has a broad specificity for a variety of PAMPs and many ligands have been identified. For example, TLR4 recognises the gram-negative bacterial product LPS. TLR2 also recognises lipoprotein from eubacteria and as a dimer with TLR6, peptidoglycan found on most bacteria. TLR3 recognises dsRNA, TLR5 recognises flagellin, while TLR9 recognises unmethylated CpG motifs in bacterial and viral DNA (Medzhitov and Janeway, 1997; Ozinsky et al., 2000) (Fig. 1.3).

The molecular events involved in TLR signalling following engagement of these receptors has been reviewed by a number of groups (Akira et al., 2001; Medzhitov, 2001; Palsson-McDermott and O'Neill, 2004). Briefly, TLRs are transmembrane proteins, with an extracellular leucine-rich motif that participates in ligand recognition and a cytoplasmic portion that contains conserved regions called the Toll/IL-1R resistance (TIR) domain, which can trigger intracellular signalling pathways (Medzhitov et al., 1998). The conserved nature of the TIR domain allows
Figure 1.3 Schematic diagram of the regulation of adaptive immune responses by TLRs

TLRs respond to specific PAMPs, utilize different adaptor proteins and induce different T helper (Th)-associated immune responses. TLR3, 7, 8 and 9 are usually found intracellularly. TLR4 requires CD14 and MD-2 in order to respond to LPS. TLR2 can dimerise with either TLR1 or 6 in order to respond to a variety of activators. Details of these interactions can be found in the text.

Abbreviations: PAMPs, pathogen associated molecular patterns; ssRNA, single-strand RNA; dsRNA, double-strand RNA; RSV, respiratory syncytial virus; LPS, lipopolysaccharide; LBP, LPS-binding protein; TLR, Toll-like receptor; Mal, MyD88-adaptor protein; TRIF, TIR domain-containing adapter-inducing IFN-β; TRAM, TRIF-related adaptor molecule.

TLRs to use the same signalling molecules as the IL-1R, including myeloid differentiation factor 88 (MyD88), IL-1R-associated protein kinase (IRAK) and tumor necrosis factor receptor-activated factor 6 (TRAF-6). The MyD88-dependent pathway, which plays a role in the early response to LPS, involves the recruitment of MyD88 to the TLR and interaction of its TIR domain with that of the TLR. Through its death domain, MyD88 recruits and phosphorylates the serine-threonine kinases IRAK 1 and 4, which, after dissociation from the receptor complex, interact with TRAF-6. TRAF-6 then becomes activated and interacts with TAB-2 (TAK-1 binding protein), which activates the mitogen-activated protein (MAP) kinase kinase kinase (MKKK) TAK-1 (transforming growth factor-β-activated kinase). TAK-1 is constitutively associated with its adaptor protein TAB-1 and is a common activator of both the MAP kinase pathway and the Rel family transcription factor nuclear factor κB (NFκB), which mediate a variety of aspects of immune regulation, including the production of the pro-inflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α and IL-18 (Bowie and O'Neill, 2000; Janssens and Beyaert, 2002; Palsson-McDermott and O'Neill, 2004) (Fig. 1.4).

However, evidence is accumulating that additional adaptor proteins associate with specific TLRs and, therefore, specific signals may be activated through distinct TLRs, resulting in different immune responses. One such adaptor protein is Mal (MyD88 adaptor like; also known as TIRAP) (Fitzgerald et al., 2001), an essential adaptor for MyD88 in LPS/TLR4 signalling (Horng et al., 2001). Recent studies from knockout mice have shown that TLR2 also utilizes this adaptor protein but signalling via TLR 5, 7 and 9 is independent of Mal (Horng et al., 2002; Yamamoto et al., 2002a). The next adaptor to be identified was TRIF (TIR domain-containing adapter inducing IFN-β) (Yamamoto et al., 2002b) and it is involved in the MyD88-
Figure 1.4 A simplified model of LPS/TLR4 signalling

Details of these interactions can be found in the text.

Abbreviations: IRAK, IL-1R-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; TAB, TAK-1 binding protein; TAK, transforming growth factor-β-activated kinase; NFκB, nuclear factor κB; NEMO, NFκB essential modulator; IκB, inhibitor of NFκB; IKK, IκB kinase; TBK, TANK-binding kinase; IRF, interferon regulatory factor; MAP, mitogen activated protein; ISRE, interferon stimulated response element, AP-1, activated protein-1.

independent response to LPS (Yamamoto et al., 2003). TRIF plays an important role in the activation of interferon-regulatory factor-3 (IRF-3) and type-1 IFNs in response to TLR3- and TLR4-mediated signalling. Indeed, TRIF is vital for TLR3 signalling (Yamamoto et al., 2003). The most recently described TLR adaptor protein, TRAM (TRIF-related adaptor molecule), is uniquely required for signalling via TLR4 and is involved in MyD88-independent activation of IRF-3 and -7 and NFκB by LPS (Fitzgerald et al., 2003).

MyD88 appears to play an important role in the development of Th1 responses; macrophages and DCs from mice deficient in MyD88 do not produce the pro-inflammatory cytokines TNF-α or IL-12 in response to CpG, LPS, flagellin or dsRNA (Adachi et al., 1998; Hacker et al., 2000; Kawai et al., 1999). Indeed, MyD88 knockout mice were shown to have impaired Th1 responses when immunized with Ag in the presence of LPS (Kaisho et al., 2002) or complete Freund’s adjuvant (CFA) (Schnare et al., 2001). However, not all signals via the TLRs result in a pro-inflammatory response. Signalling via TLR2 does not result in the production of the Th1-promoting factors IL-12p70 or interferon-γ inducible protein (IP)-10 (Hirschfeld et al., 2001). In fact, activation of DCs via TLR2 induces abundant levels of IL-10, little IL-12p70 and modulates the response toward the Th2 pathway in vivo (Dillon et al., 2004; Re and Strominger, 2001) (Fig. 1.3).

1.3.3 Costimulatory molecules

Optimal T cell activation requires a minimum of two separate signals; one via engagement of the TCR, and the other though costimulation molecules. The most common costimulatory pathway is the B7/CD28 pathway. CD80 (B7-1) and CD86 (B7-2) are found on multiple cell types, including macrophages, activated B- and T-
cells, DCs and activated monocytes (Hathcock et al., 1994). This family plays an important role in the initiation of the adaptive immune response and their upregulation on DCs is used as a marker for maturation. A number of studies have suggested that CD80 and CD86 may play distinct roles in Th cell differentiation. An examination of the role of these costimulatory molecules during experimental allergic encephalomyelitis (EAE) suggested that CD86 costimulation directs T cell activation towards a Th2 phenotype while CD80 signalling directs Th1 cell development (Kuchroo et al., 1995). In addition, through blocking CD86 on T helper precursor cells with specific monoclonal antibodies, this costimulatory molecule was shown to be essential for the production of the Th2 cytokine IL-4 in a secondary stimulation, whereas the Th1 cytokines, IL-2 and IFN-γ, were not affected (Ranger et al., 1996).

Other surface molecules, including CD40, intracellular adhesion molecules (ICAM) and OX40L, can also modulate the type of Th response induced by an APC. CD40, a member of the TNF receptor family, is found on all APCs including DCs (Inaba et al., 1994). It interacts with CD40 ligand (CD40L), another member of the TNF family, which is expressed mainly on CD4+ Th cells (Roy et al., 1993). CD40-CD40L interaction results in the induction of IL-12 from the DCs (Cella et al., 1996). Ligation of CD40 also results in increased expression of IL-1β, IL-1R antagonist (IL-1Ra), IL-8, regulated on activation normal T cell expressed and secreted (RANTES), MIP-1α and monocyte chemoattractant protein-1 (MCP-1) (Aicher et al., 1999) and indirectly enhances survival of activated CD4+ Th cells, thereby promoting a more robust immune response (Maxwell et al., 1999). Therefore, CD40-CD40L interaction is an important process in the generation of mature DCs that polarise T cells towards a Th1 phenotype.
ICAMs are constitutively expressed on a number of cell types, including some lymphocytes and monocytes, but their expression can be significantly increased in the presence of inflammatory cytokines or certain microbial products (e.g. LPS) (Hubbard and Rothlein, 2000). ICAMs are ligands for the β2 integrin molecules, lymphocyte function-associated antigen 1 (LFA-1 or CD11a/CD18) and MAC-1 (CD11b/CD18). ICAM-1 (CD54), the most extensively studied of the five ICAMs discovered to date, binds LFA-1 during Ag presentation and plays an important role in the activation of T cells. Studies have shown that ligation of LFA-1 on T cells by Fc-ICAM-1 or anti-ICAM-1 antibodies, skewed the differentiation of naïve Th cells towards the Th1 phenotype, through the suppression of Th2-associated cytokine production (Salomon and Bluestone, 1998), via a direct, IL-12-independent mechanism (Smits et al., 2002).

OX40L, which is expressed on B cells and DCs (Ohshima et al., 1997) interacts with its receptor, OX40 on activated T cells, increasing cell cycle turnover and thereby prolonging the longevity of these T cells (Weatherill et al., 2001). OX40-OX40L interaction has also been implicated in enhancing Th2 differentiation from naïve T cells, through the induction of IL-4 production and inhibition of IFN-γ expression in IL-12-stimulated CD4⁺ Th cells (Flynn et al., 1998).

1.4 Th cell polarization

1.4.1 Role of cytokines in Th1 cell development

The differentiation of naïve Th cells to distinct T cell subsets can be influenced by a number of factors, including the APC and/or co-stimulatory molecules, and the nature and dose of Ag. However, the dominant regulators in T cell differentiation are undoubtedly cytokines (Abbas et al., 1996; Constant and Bottomly, 1997). One of the most extensively studied cytokines involved in the differentiation
of Th1 cells is IL-12. In addition to directing Th1 responses, IL-12 also enhances phagocytosis and bactericidal activity of other APCs, thereby forming a link between innate and adaptive immunity (reviewed in Ma and Trinchieri, 2001). IL-12 primes naïve CD4+ Th cells for high IFN-γ production, which plays a critical role in protection against intracellular pathogens (Hsieh et al., 1993; Mosmann and Sad, 1996; Trinchieri, 1994). DCs are the prime source of this cytokine following exposure to microbial stimuli (Macatonia et al., 1995; Sousa et al., 1997).

The production of IL-12 is initiated by numerous stimuli, including engagement of TLRs by microbial ligands (discussed above), ligation of either CD40 or MHC class II on the surface of DCs (Koch et al., 1996) and IFN-γ induced by natural killer (NK) cells and activated T cells (Trinchieri, 1989). Indeed, DCs themselves have been shown to produce IFN-γ in response to IL-12 (Fukao et al., 2000), indicating an autocrine feedback loop that further propagates a Th1-inducing cytokine environment. However, the initial production of IL-12 occurs rapidly and is independent of T-cell derived signals (Gazzinelli et al., 1994).

Bioactive IL-12 is a heterodimer cytokine composed of disulfide-linked p40 and p35 chains. The p40 chain is produced in vast excess over p35 and can be secreted as homodimers, and studies in mice have suggested that these homodimers may be natural inhibitors of bioactive IL-12 (Gillessen et al., 1995). Expression of p35 is the rate-limiting step in the production of IL-12 (Snijders et al., 1998), but formation of the heterodimer requires additional post-translation modification. The IL-12 receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2, which activate the Janus kinases, JAK1 and TYK2, which then phosphorylate the signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4 and STAT5 (Presky et al., 1996). The production of high levels of bioactive IL-12 (IL-12p70) by DCs appears
to require two signals; a priming signal provided by for example IFN-γ or GM-CSF and a second challenging signal, which can be mediated by bacterial products, including LPS, bacterial DNA, heat shock proteins or intracellular parasites (Ma et al., 1996; Snijders et al., 1998). In contrast, IL-12p70 can be negatively regulated by a wide variety of factors, including prostaglandin E2 (PGE2), IL-10, IL-4 and elevated levels of cyclic 3', 5'- adenosine monophosphate (cAMP) (Feng et al., 2000; Karp et al., 1998; Van der Pouw-Kraan et al., 1995).

The regulation of the gene encoding p40 has been examined in some detail but less is know about the induction of p35. A number of important transcription factors play a role in IL-12p40 gene transcription, including NFκB, C/EBP (CCAAT/enhancer-binding protein) and activator protein (AP)-1, which appear to be downstream of TLR signalling, as well as Ets2, IRF-1 and IFN-consensus sequence-binding protein (ICSBP) which are activated by IFN-γ and STAT1/2 signalling (Trinchieri, 2003). A repressor site in the promoter region of p40, known as GA12 (GATA sequence in the IL-12 promoter) has been shown to be essential for the early inhibition of IL-12p40 by IL-4 or PGE2 (Becker et al., 2001). Transcription of the p35 gene is associated with transcription factors such as SP1, IFN-γ-response element (γ-IRE), PU.1 and C/EBP (Yoshimoto et al., 1996). In addition, expression of p35 requires posttranslational regulation (Babik et al., 1999).

IL-23 and IL-27, two other members of the IL-12 cytokine family, have recently been described (Oppmann et al., 2000; Pflanz et al., 2002). IL-23 is composed of the p40 subunit of IL-12, which associates with another molecule, p19. IL-27 consists of EBI3 (Epstein-Barr virus-induced gene 3), which is related to p40 and a novel helical subunit, p28. IL-27 induces proliferation and IFN-γ production in naïve T cells only, IL-12 induces significant proliferation and IFN-γ secretion in both
naïve and memory T cells, while IL-23 has no effect on naïve T cells but induces proliferation and IFN-γ from activated memory T cells (Robinson and O'Garra, 2002). Therefore, these three cytokines appear to have sequential contributions to the development and maintenance of Th1 responses.

The interferons (IFNs) are a family of cytokines with potent antiviral activity and play an important role in the early response to pathogens. Type I IFNs comprise at least four subtypes (IFN-α, IFN-β, IFN-ω and IFN-τ) and share a common two-chain receptor (IFNαR1 and IFNαR2). Type II IFNs are represented by a single cytokine (IFN-γ), which binds to a distinct receptor, composed of two chains (IFNγR1 and IFNγR2) (Pestka, 2000; Pestka et al., 1997). IFN-α and IFN-β genes are induced rapidly, through a mechanism involving the transcription factors IRF-7 and IRF-3 respectively (Hiscott et al., 1999), in response to a variety of bacterial components as well as viral particles (reviewed in Le Bon and Tough, 2002). Engagement of the type I IFN receptor activates the JAK-STAT pathway (particularly STAT1), which regulates the expression of IFN-stimulated genes (ISGs) via the IFN-stimulated response element (IRSE) in their promoters (Stark et al., 1998). IFN-α/β can induce the maturation of myeloid DCs (Ito et al., 2001). However, type I IFNs can have opposing effects on the generation of IFN-γ-secreting Th1 cells. Maturation of immature DCs in the presence of IFN-β results in the strong augmentation of Th1 responses, possibly through the enhancement of IL-18. However, the presence of IFN-β during mature DC-mediated primary stimulation of naïve T cells significantly inhibited Th1 cell development (Nagai et al., 2003). In addition, high levels of IFN-α/β negatively regulates IL-12 production, decreasing both p40 chain and the p70 heterodimer (Cousens et al., 1997). The inhibition of IL-12 by high levels of IFN-α/β may be necessary to preferentially promote antiviral defences through CD8⁺ T cell
responses, NK cell cytotoxicity and the further augmentation of IFN-α/β over IFN-γ production.

The role of IFN-γ in T cell responses appears to involve the induction of stable, polarised effector DCs with enhanced IL-12-producing capacity (Vieira et al., 2000) and the enhancement of the IL-12Rβ2 subunit on T cells, making them more responsive to this cytokine (Gillespie et al., 2000). In addition to the involvement of IL-12 and IFN-γ in the differentiation of Th1 cells, IL-12 also acts in synergy with another IFN-γ-inducing factor, IL-18. This cytokine shares many biological functions with IL-12, including the induction of IFN-γ production from Th1 cells and NK cells (Micallef et al., 1996). However, the function of IL-18 appears to be completely dependent on IL-12 production and therefore appears to acts by augmenting an IL-12-driven Th1 response (Micallef et al., 1996; Robinson et al., 1997).

1.4.2 Role of cytokines in Th2 polarization

IL-4 has the greatest influence on promoting the differentiation of naïve precursor T cells towards Th2 cells (Swain et al., 1990). When both IL-12 and IL-4 are present together in the same culture, the effect of IL-4 is dominant and Th2 cells develop (Hsieh et al., 1993). The early source of IL-4 that initiates the development of Th2 cells remains to be clearly defined. Cells of the innate immune system, including mast cells, eosinophils, NK cells and NK T cells may serve as an early source of IL-4 (Brown et al., 1987; Moqbel et al., 1995; Seder et al., 1994). However, cross-linking of the TCR on naïve T cells, in the presence of CD28 costimulation, dramatically increases the generation of Th2 cells through an IL-4-mediated pathway (Kubo et al., 1999) suggesting that Th2 differentiation can be supported solely by IL-4 production by Ag-primed CD4+ T cells. In addition, IL-4 downregulates the β2
subunit of the IL-12 receptor on T cells, thereby reducing IL-12-driven Th1 cell development (Wu et al., 2000).

In addition to IL-4, IL-6, which is produced by macrophages and DCs in response to TLR ligation, can polarise naïve CD4\(^+\) T cells to effector Th2 cells. IL-6 directly interacts with the T cell to upregulate IL-4 gene expression, which may be sufficient to initiate a positive autocrine feedback loop for IL-4 production, resulting in the polarization of the cells to a Th2 phenotype, through their own IL-4 (Rincon et al., 1997). IL-6 also directly inhibits IL-12 production thereby inhibiting Th1 cell development (Dodge et al., 2003).

1.4.3 Cytokines and Tr1 cell development

Although inflammation is an essential host response to infectious challenge, the response must be tightly regulated. One mechanism involved in the avoidance of damage to the host is achieved by through direct suppression mediated by Tr cells. One population of Tr cells, Tr1 cells, function as suppressor T cells through their production of IL-10 and TGF-β (Barrat et al., 2002; Groux et al., 1997). IL-10, which is produced by Th2 cells as well as Tr1 cells, is an important cytokine involved in the suppression of inflammatory responses, inhibiting monocyte/macrophage/DC activation and the expression of inflammatory mediators, including the expression of IL-12p35 and IL-p40 via the synthesis of an as-yet-unidentified protein (Aste-Amezaga et al., 1998). An essential regulatory role of IL-10 was shown by the uncontrolled, lethal, systemic inflammatory response seen in IL-10 knockout mice infected with *Toxoplasma gondii* (Gazzinelli et al., 1996). IL-10 also has a role in immune tolerance and the differentiation of B cells (Akbari et al., 2001; Go et al., 1990; Rousset et al., 1992). As a result of its anti-inflammatory properties, IL-10 has
been assessed as a therapeutic agent for inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel disease.

Engagement of the IL-10R results in the activation of the JAK-STAT pathway with the predominant activation of STAT3 (Moore et al., 2001), which is a requirement for many of the anti-inflammatory effects of IL-10 (Riley et al., 1999). IL-10 also induces suppressor of cytokine signalling-3 (SOCS-3) (Cassatella et al., 1999), which has previously been shown to inhibit IFN-inducible gene expression through suppression of STAT1 phosphorylation (Terstegen et al., 2000). Since IFNs are important mediators of protective immunity against infection, inhibition of the signalling molecules involved in IFN-induced signal transduction (e.g. STAT1) by IL-10 may be a mechanism employed by certain infectious agents to prolong their survival in the host.

However, the regulation of the synthesis of IL-10 is not well understood. AP-1, cAMP-responsive element binding protein (CREB), Sp1 and Sp3 have been reported to participate in the induction of IL-10 gene expression (Brightbill et al., 2000; Platzer et al., 1999). Numerous studies have suggested that agents that elevate cAMP are involved in the enhancement of IL-10 production from monocytes, but the mechanism involved has not yet been described (Feng et al., 2000; Zidek, 1999). STAT3 has also been associated with LPS-induced IL-10 production (Benkhart et al., 2000). In addition, IRAK1 has been shown to play an important role in LPS-induced IL-10 production in a STAT-3-dependent manner (Huang et al., 2004). Huang et al. demonstrated that IRAK1 is essential for STAT3 serine phosphorylation in response to LPS. IRAK1 also forms a complex with STAT3 and directly interacts with the promoter region of the human IL-10 gene. Therefore, IRAK1 not only appears to
regulate IL-10 gene expression through the activation of STAT3, but also may
directly serve as a transcriptional regulator for IL-10 gene transcription.

1.4.4 Molecular events involved in Th polarization

Activation of the Th1 cytokine, IFN-γ, is regulated by a number of signalling
pathways and transcription factors. Firstly, STAT4 is an important determinant in
IFN-γ production, as illustrated through the use of STAT4-deficient mice, which have
impaired IFN-γ responses, and as a consequence defective Th1 development (Kaplan
et al., 1996). STAT4 is the predominant STAT member activated through the IL-12R
on the surface of T cells (Jacobson et al., 1995) and is required for the induction of
IFN-γ by IL-12. However, differentiated Th1 cells are capable of producing IFN-γ at
high levels in the absence of STAT4 (Yang et al., 1999), therefore additional
signalling molecules must be involved. IL-18, which synergises with IL-12 in
stimulating IFN-γ, activates IRAK and NFκB in Th1 cells, which acts on discrete cis-
acting elements in the IFN-γ regulatory region (Kojima et al., 1998). A novel Th1-
specific transcription factor, T-bet (T-box expressed in T cells), has been reported to
play a role in the expression of IFN-γ, through binding to the promoter of this gene
(Szabo et al., 2000; Szabo et al., 2003). Induction of T-bet, a member of the T-box
family of transcription factors, is heavily dependent on STAT1, which is induced by
IFNs, and is independent of STAT4 (Afkarian et al., 2002). Retroviral infection of
primary CD4+ T cells with T-bet resulted in the dramatic increase in IFN-γ producing
cells. In addition, introduction of T-bet into developing Th2 cells reduced the number
of IL-4 producing cells (Szabo et al., 2000). T-bet can also induce IL-12Rβ2
expression thereby making the cells more responsive to IL-12 (Afkarian et al., 2002).
However, T-bet needs to be expressed above a certain threshold to achieve significant
induction of IFN-γ (Szabo et al., 2000). The transcription factor ERM, a member of the Ets family, is selectively expressed in Th1 cells and is induced by IL-12 in a STAT4-dependent manner (Ouyang et al., 1999). However, ERM does not directly induce IFN-γ production, indicating that it may cooperate with additional factors such as STAT4 in the regulation of IFN-γ. Alternatively ERM may not be involved in IFN-γ production by Th1 cells but may play a role of IFN-γ-independent Th1 development. Clearly, polarisation of Th1 cells by IL-12 and IL-18 requires a number of different factors for transcription of the IFN-γ gene (Fig. 1.5).

IL-4-induced differentiation of CD4⁺ T cells to Th2 cells involves STAT6 and mice deficient in IL-4 or STAT6 have impaired Th2 responses (reviewed in Abbas et al., 1996). A STAT6 binding site in the Th2 locus has not been identified to date. However, STAT6 induces the expression of two other Th2-specific factors, c-musculoaponeurotic fibrosarcoma (c-Maf) and GATA-3 (Kurata et al., 1999), suggesting that STAT6 acts indirectly in Th2 cell development. In addition, STAT6 down-regulates IL-12Rβ2 expression on T cells, thereby reducing their responsiveness to IL-12 (Kurata et al., 1999). c-Maf, described as the first Th2-specific transcription factor to bind directly to the IL-4 proximal promoter at a c-Maf response element (MARE) (Ho et al., 1996), exerts a very selective effect on the IL-4 gene but not on other Th2-specific genes (Kim et al., 1999). This transcription factor probably synergises with AP-1 and nuclear factor of activated T cells (NFAT) (Ho et al., 1996; Li et al., 1999), which are downstream of TCR-signalling. Interestingly, c-Maf has been recently shown to be a potent activator of the anti-inflammatory gene IL-10 (Cao et al., 2002). In addition, overexpression of c-Maf inhibited the activation of IL-12p40 and p35 genes in macrophages, indicating a pivotal role for this transcription factor in the modulation of Th responses. However, IL-10 inhibition of
Figure 1.5 The molecular basis of T helper cell differentiation

Engagement of the IL-12R results in activation of STAT4, which is involved in IFN-γ production and in the differentiation of Th1 cells via ERM. STAT1, a downstream effector of IFNR activation, is an important activator of the Th1-associated transcription factor T-bet which promotes IFN-γ and suppresses IL-4 production. IL-18R-induced enhancement of IFN-γ involves IRAK and NFκB. Engagement of the IL-4R activates STAT6, which is upstream of the transcription factors c-Maf and GATA-3. These transcription factors are involved in the induction of the Th2-associated cytokines IL-4, IL-10 and IL-5. In addition, GATA-3 suppresses Th1 cell development. TCR activation induces NFAT2 and AP-1, which promote IL-4 production. Abbreviations: IL-18R, interleukin-18 receptor; IFNR, interferon receptor; TCR, T cell receptor; STAT, signal transducer and activator of transcription; NFAT, nuclear factor of activation in T cells; IRAK, IL-1R-associated kinase; AP, activated protein; T-bet, T-box expressed in T cells.
IL-12 is intact in the absence of c-Maf, but appears to be dependent on endogenous AP-1.

GATA-3 is a Th2-specific transcription factor, which is induced by IL-4 in a STAT6 dependent manner (Ferber et al., 1999). It promotes expression of several Th2 cytokines, including IL-4 and IL-5 (Zheng and Flavell, 1997) and can directly inhibit the production of IFN-γ in a mechanism independent of IL-4 and STAT6 (Ferber et al., 1999; Ouyang et al., 2000; Ouyang et al., 1998), possibly through the downregulation of STAT4 (Usui et al., 2003). Expression of GATA-3 in developing but not committed Th1 cells induces the expression of endogenous GATA-3, suggesting an autoregulative loop for GATA-3 in Th2 cells (Lee et al., 2000).

NFAT and AP-1 also play a role in Th2-associated cytokine production. Activation of NFAT requires both protein kinase C (PKC) and Ca$^{2+}$ signals and induction of NFAT transcriptional activity requires TCR-mediated signals and co-stimulatory signals (De Boer et al., 1999). NFAT appears to play a critical role in the transcriptional regulation of IL-4, with both NFAT1 (NFATp or NFATc2) and NFAT2 (NFATc or NFATc1) directly binding to the promoter sequence of the IL-4 gene (Yoshida et al., 1998). NFAT also cooperates with AP-1 in IL-4 gene transcription, with the composition of AP-1 differing depending on the signal and cell type (De Boer et al., 1999). NFAT2 positively regulates IL-4 production in a STAT6-independent manner, while NFAT1 suppresses GATA-3 expression and inhibits early IL-4 production. Therefore, by altering the relative levels of the two transcription factors NFAT1 and NFAT2, the potency of TCR signalling can positively or negatively modulate IL-4 production, and therefore influence the Th1/Th2 balance (Brogdon et al., 2002). Members of the MAP kinase signalling family are also involved in polarization of Th subsets and are discussed below.
1.5 MAP kinase signalling family

The MAP kinase signalling cascade connects cell-surface receptors to critical regulatory targets within cells. MAP kinases respond to a variety of stimuli, including chemical and physical stresses, growth factors and cytokines, thereby controlling cell survival and adaptation. The generation of mice with target mutations and the development of specific inhibitors have revealed that the MAP kinases are involved in almost all cellular processes to some degree, from gene expression to cell death.

The MAP kinase cascade is composed of at least three major groups of kinases in mammalian cells – the extracellular signal-related protein kinases (ERK1/2), the p38 MAP kinases (p38α,β,γ,δ) and the c-jun NH₂-terminal kinases (JNK1/2/3). All three family members are activated through tyrosine and threonine phosphorylation of a common Thr-X-Tyr regulatory motif in the activation loop of the kinase by their distinct upstream dual-specificity (Thr/Tyr) MAP kinase kinase (Kyriakis and Avruch, 2001). This tri-peptide motif is different in each of the MAP kinases and is phosphorylated by a conserved protein kinase cascade, referred to as MAP/ERK-kinases (MEKs) or MAP kinase kinase (MKKs) (Fig. 1.6).

ERK is activated by MEK1 and MEK2; p38 by MKK3, MKK4 and MKK6 while MKK4 and MKK7 activates JNK. The MEKs or MKKs are in turn phosphorylated by MKKK (Dong et al., 2002). The flexibility of the MAP kinases to respond to a wide range of cellular stimuli is provided by the diversity of the regulatory domains in different MKKs.

1.5.1 Extracellular signal related kinase (ERK) pathway

The ERK pathway is the best-characterised of the MAP kinase pathways and participates in cell proliferation, apoptosis and a number of other important cellular
Figure 1.6 The mitogen activated protein (MAP) kinase signalling pathways

Various stimuli lead to the activation of the MAP kinase signalling cascade, which consists of core MAPK kinase kinase (MKKK) → MAPK kinase (MKK/MEK) → MAPK pathways, which then activate various transcription factors. Three major groups of kinases exist, the extracellular signal related protein kinases (ERK1/2), p38 MAP kinases (p38α,β,γ,δ) and the c-jun NH2-terminal kinases (JNK1/2/3). Each group is activated by tyrosine and threonine phosphorylation by specific M KK. 

Abbreviations: MSK, mitogen and stress activated protein kinase; STAT, signal transducer and activator of transcription; MAPKAP-K, MAPK activated protein-kinase; ATF, activating transcription factor; NFAT, nuclear factor of activated T cells. Adapted from Kyriakis and Avruch, 2001.
responses (Graves et al., 1995). ERKs are believed to respond to mitogens and growth factors, in addition to a variety of other stimuli, that regulate cell proliferation and differentiation (Lewis et al., 1998). The Ras-Raf-MEK-ERK pathway is the classical pathway leading to ERK activation. Upon activation by mitogens and growth factors, their receptors recruit the small guanosine triphosphatase (GTPase), Ras. Ras activates the serine/threonine kinase Raf (a MKKK), which in turn activates MEK1 and MEK2. These MKKs phosphorylate ERK1 (44kDa) and ERK2 (42kDa), which transactivates a variety of transcription factors. Some of the transcription factors phosphorylated by ERK1 and ERK2 are Elk-1, mitogen- and stress-activated protein kinases (MSKs), Ets1, STATs, MAPKAP-K1 (RSK), sos (which activates Ras) and the regulatory enzyme phospholipase A2 (Garrington and Johnson, 1999).

A number of studies have examined the role of ERK1/2 in the activation and regulation of APCs and T cells. The role of ERK in macrophage and DC function remains unclear but recent studies have highlighted its importance in the initiation of diverse responses in these cells. LPS has been shown to activate ERK phosphorylation in macrophages (Feng et al., 1999) and DCs (Rescigno et al., 1998). Activation of DCs by TNF-α also involves phosphorylation of ERK kinase (Sato et al., 1999). The mechanism of LPS activation of ERK has yet to be fully understood, however a number of signalling molecules appear to be involved. LPS-induced PKC-ζ has been shown to play an important role in the activation of ERK through MEK phosphorylation in macrophages (Monick et al., 2000). Raf1, a MKKK, which binds to activated Ras protein and phosphorylates MEK, may also be involved in LPS-induced ERK activation in macrophages (Reimann et al., 1994) and DCs (Nakayama et al., 2003).
ERK, through activation of RSK, can modulate cell survival by positively regulating the transcription of pro-survival genes, including the Bcl-2 family members, in a variety of cell types (reviewed in Ballif and Blenis, 2001). LPS-induced activation of DCs involves both maturation of the cell and rescue from apoptosis after withdrawal of growth factors. Inhibition of ERK resulted in a dramatic reduction of LPS-induced cell survival (Rescigno et al., 1998) suggesting a role for ERK in LPS-induced cell survival. In addition, ERK activation in response to cAMP has recently been associated with the transient inhibition of apoptosis in the intestinal crypt-like cell line, T84 cells (Rudolph et al., 2004) possibly through the induction of cellular inhibitor of the apoptosis protein-2 (c-IAP2) (Nishihara et al., 2004).

Studies with specific inhibitors to ERK activation have highlighted a role for this kinase in the suppression of LPS-mediated IL-12 production in both macrophages (Feng et al., 1999) and in DCs (Puig-Kroger et al., 2001). This suppression of IL-12 production was shown to involve the inhibition of IRF-1, which is involved in the transcription of IL-12p40 in macrophages (Goodridge et al., 2003). It has also been shown that LPS-induced TNF-α production, and possibly IL-10, is regulated, at least in part, by ERK. Stimulation of monocytes with LPS in the presences of ERK inhibitors resulted in the significant reduction of both secreted levels of TNF-α and in its mRNA (Foey et al., 1998; van der Bruggen et al., 1999). The role of ERK in IL-10 production remains to be fully elucidated. The ERK inhibitor PD98059 was found to have no effect on LPS-induced secretion of IL-10 by monocytes (Foey et al., 1998). In contrast, ERK has been shown to be essential for the production of IL-10 by CpG (which binds to TLR9) in a macrophage-like cell line, RAW 264.7 (Yi et al., 2002),
suggesting the possibility that ERK may play a role in the regulation of IL-10 production from certain TLRs but not others.

Phosphorylation of ERK is an important event in the activation of T cells (summarized in Fig. 1.7). Upon engagement of TCR, various signal transduction molecules are recruited to the point of activation, including Grab2 and SLP76. These molecules activate the sos-Ras-MEK-ERK pathway. ERK1 has been shown to be vital for thymocyte development, as ERK1−/− mice display defective thymocyte maturation (Pages et al., 1999). ERK activation, through TCR engagement, is also involved in the Th2 differentiation of CD4+ T cells in the periphery (Yamashita et al., 1999). This study showed that dominant negative Ras transgenic mice had defective Th2 cell development, and also suggested that the ERK pathway functioned to enhance IL-4-induced STAT6 and IL-4R phosphorylation.

1.5.2 The p38 MAP kinase

The p38 MAP kinase is activated by stress, differentiation factors, inflammatory cytokines and growth factors. The transcription factors phosphorylated by p38 include AFT-2, CHOP, MEFC2, NFAT-1, MSK, MAPKAP-K2/3 and Elk-1 and this kinase is necessary for the activation of c-Jun and c-fos responses to anisomycin and UV irradiation (Garrington and Johnson, 1999; Tibbles and Woodgett, 1999). p38 is phosphorylated by three different MKKs; MKK3, MKK4 and MKK6. MyD88 and other molecules in the NFκB activation pathway are also involved in the activation of p38. MyD88 knockout mice are defective in LPS-induced MAP kinase activation (Kawai et al., 1999) and TRAF6+/− and IRAK+/− cells exhibit deficiencies in p38 and JNK activation in response to IL-1 (Kanakaraj et al., 1998; Lomaga et al., 1999). TAK-1, which is downstream of these signalling
JNK does not appear to be necessary for IL-2 production from naïve CD4\(^+\) T cells. ERK 1/2 activation is required for Th2 cell development. JNK1 is a negative regulator of Th2 differentiation. Both JNK2 and p38 are involved in the production of interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β) and the polarization of Th1 cells. p38 also regulates the production of IFN-γ (and other effector molecules) from CD8\(^+\) cells, and is involved in the death of these cells.

Reproduced from Rincon, 2001
molecules, can activate MKK3 and MKK6, thereby providing a link between TLR/IL-1R signalling pathway and p38 activation (Kyriakis and Avruch, 2001). In addition, the small G protein, Ras, plays a role in the activation of p38 in response to IL-1 (Palsson et al., 2000).

P38 plays an important role in IL-12 production by macrophages and DCs. MKK3<sup>−/−</sup> cells showed a selective defect in IL-12 production when stimulated by LPS, although concentrations of IL-1α, IL-1β, TNF-α and IL-6 were comparable with the wild-type cells (Lu et al., 1999). Interestingly, p38 specific inhibitors abrogated IL-1α and IL-1β as well as IL-12 production, suggesting that the MKK3-p38 pathway plays a specific role in LPS-induced IL-12 regulation, while other MKKs may compensate during IL-1-induced IL-12 production in macrophages and DCs. Furthermore, the activation of p38 in response to sorbitol was similar in wild-type and MKK3<sup>−/−</sup> macrophages illustrating the specificity of MKK3-p38 activation in LPS-induced IL-12 production in macrophages (Lu et al., 1999).

A number of studies have shown that inhibition of p38 results in defective LPS-induced IL-12 production in macrophages (Feng et al., 1999) and DCs (Puig-Kroger et al., 2001) and complete inhibition of CD40-induced IL-12 production in DCs (Aicher et al., 1999). Therefore, it is possible that p38 and ERK may play opposing roles in the regulation of IL-12 production from innate cells. However, p38 may have a negative effect on IFN-γ-dependent IL-12p40 production in peripheral blood mononuclear cells (PBMCs), with inhibition of p38 resulting in the significant augmentation of IL-12p40 (Marriott et al., 2001), suggesting a possible distinct role for p38 in IL-12 production, depending on the stimulus and cell type.

P38 MAP kinase may also play a role in the regulation of other cytokines from DCs and macrophages. Inhibitors of p38 suppress IL-10 production from LPS-
stimulated human monocytes (Lim et al., 2002; Ma et al., 2001) or from CpG-activated murine macrophages (Yi et al., 2002), as well as TNF-α and IL-1β production by LPS-stimulated PBMCs (Foey et al., 1998). Indeed, IL-10 production from cells from patients with schistosomiasis has been shown to involve the activation of p38 and subsequently the transcription factor ATF-2 (Cameiro-Santos et al., 2002). p38 also plays a role in the upregulation of TLR2 and TLR4 expression in response to LPS stimulation (An et al., 2002). The phosphorylation of the transcription factor CREB (cAMP-responsive element binding protein) by CpG was shown to be dependent on the activation of p38 (Yi et al., 2002). p38 also plays a critical role in the maturation of DCs by LPS and TNF-α, with inhibition of maturation observed in the presence of the p38 inhibitor, SB203580 (Arrighi et al., 2001).

In Th cell activation, p38 plays a pivotal role in the selective induction of Th1 cells. Inhibitors of p38 and dominant-negative p38 mutants selectively impair Th1 responses (Rincon et al., 1998). This impairment was characterised by the inhibition of IFN-γ, without an effect on IL-4 production. Furthermore, MKK3−/− mice have diminished Th1 responses, primarily due to decreased IL-12 production by macrophages and DCs, but also due to impaired IL-12-induced Th1 development, implying a role for p38 in Th1 cell development (Lu et al., 1999). However, the role of p38 in T cells does not appear to be solely confined to Th1 cells. In Th2 cells, cAMP-induced events seem to be mediated by p38, resulting in the promotion of IL-5 and IL-13 gene expression (Chen et al., 2000). This appears to be a cell selective event however, as cAMP barely increases p38 activity in Th1 cells. Furthermore, the critical transcription factor of Th2 cytokine production, GATA-3, was shown to be phosphorylated by p38. Therefore, it is evident that p38 plays a role in selection of
Th1 cells, but may also be involved in augmenting cytokine production from committed Th2 cells.

1.5.3 c-Jun N-terminal kinase (JNK)

JNK is another member of the MAP kinase family that is activated by stress, growth factors, differentiation factors and inflammatory cytokines. Three JNK genes have been identified in mammalian cells, *Jnk1, Jnk2, and Jnk3* (Ip and Davis, 1998). JNK1 and JNK2 are widely expressed in a number of tissue groups, whereas JNK3 is more selectively expressed in brain, testis and heart. JNK is activated by two MKKs, MKK4 and MKK7 and acts on a number of transcription factors including c-Jun, AP-1, ATF-2, NFAT2, NFAT4 (Ip and Davis, 1998). JNK activity can be strongly induced in multiple cell types by LPS or inflammatory cytokines such as TNF-α and IL-1 (Chang and Karin, 2001; Davis, 2000) but the function of JNK in innate cells has still to be fully elucidated. JNK is activated by TRAF-2 in the TNF signalling pathway (Lee et al., 1997) and by TRAF-6 in the IL-1 pathway (Lomaga et al., 1999). Studies in MKK7 knockout cells have suggested that MKK7 is essential for activation of JNK in response to these inflammatory cytokines (Tournier et al., 2001). However both MKK7 and MKK4 are involved in activation of JNK following physical stress (Tournier et al., 2001). In addition to a role in TNF signalling pathway, JNK is also necessary for enhancement of LPS-induced TNF-α gene expression and translation (Karin et al., 1997).

The role of JNK in T cell development has been well investigated. JNK and AP-1 activation in T cells requires both TCR/CD3 and the co-stimulatory molecule CD28 (Rincon and Flavell, 1994). Studies in JNK1−/− mice has highlighted the role for this kinase in the inhibition of Th2 cell development (Dong et al., 1998). Ablation of JNK1 enhanced IL-4 production but had no effect on IL-2 or IFN-γ production.
when T cells were activated under in vitro conditions that did not drive either Th1 or Th2 cell differentiation. Further studies using JNK1−/− mice demonstrated that the inhibitory effect on Th2 cytokine production by JNK1 was essential for protective immune responses in vivo against intracellular pathogens, such as Leishmania major (Constant et al., 2000). JNK2 deficient T cells expressed a lower level of IL-12Rβ2, with decreased IFN-γ production, suggesting a role for JNK2 in Th1 cell development (Yang et al., 1998). Taken together these studies highlight a role for JNK in the polarization of T cells towards a Th1 phenotype.

1.6 cAMP

cAMP is formed through dephosphorylation of ATP (adenosine triphosphate) by the enzyme, adenylyl cyclase. Intracellular accumulation of cAMP may result from stimulation of adenylyl cyclase by a variety of molecules including β2-adrenoceptor agonists (Barnes, 1995), histamine H2 receptors (Hill, 1992), or by arachidonic acid metabolites, such as prostacyclin or prostaglandins (West et al., 1991). Its activation is regulated through activation of the adenosine receptors – A1, A2 and A3, which are coupled to the guanine-nucleotide binding proteins (G proteins), Gs and Gi. Stimulation of A2 activates Gs proteins, resulting in stimulation of adenylyl cyclase, while stimulation of A1 and A3 activates Gi proteins, resulting in the inhibition of adenylyl cyclase (Palmer et al., 1995). cAMP exerts its effects by activating the cAMP-dependent protein kinase, protein kinase A (PKA), which subsequently phosphorylates downstream effector proteins such as myosin L chain kinase and CREB (Zidek, 1999). CREB associates with the cAMP binding protein (CBP), which interacts with the cAMP response element (CRE) in the promoters of cAMP-responsive genes and activates transcription. In addition to cAMP, CREB is activated by Ca2+, growth factors and stress signals, which involves p38 kinase,
calcium/calmodulin-dependent protein kinase (CaMk) IV and ERK kinase (Yu et al., 2001). CD3/CD28-induced CREB-CBP interaction involves the coordinated activation of p38, ERK and CaMk IV (Yu et al., 2001).

cAMP appears to have a role in a number of immune responses. Of interest to this study is the role of cAMP in the modulation of cytokine production by cells of both the innate and adaptive immune system, since CT is a potent enhancer of intracellular concentrations of cAMP through ADP-ribosylation of Gsα (Rappuoli et al., 1999). Normal intracellular levels of cAMP do not induce NFκB, but elevations in cAMP concentrations inhibits its activity in T cells (Chen and Rothenberg, 1994). A number of studies have shown that elevation of intracellular cAMP results in enhancement of IL-6 and IL-10 production with inhibition of IL-12, TNF-α, IFN-γ and IL-2 production by both innate cells and T cells (reviewed in Zidek, 1999). cAMP-elevating agents, such as β2-agonists, 8-bromo-cAMP and PGE₂ inhibit CD40L- and IFN-γ-induced IL-12 production by human DCs (Braun et al., 1999; Panina-Bordignon et al., 1997), as well as TNF-α release, and suppress Ag-presentation and decreases MHC class II expression on DCs (Kambayashi et al., 2001). These modulatory activities were associated with enhanced IL-10 production, suggesting that they may be mediated through IL-10.

In effector CD4⁺ T cells, elevation of intracellular cAMP is associated with inhibition of Th1 cytokine production and augmentation of IL-5 gene expression in Th2 cells (Lacour et al., 1994; Munoz et al., 1990). A recent report has demonstrated a link between cAMP, p38, GATA-3 and IL-5 production in Th2 cells, which is independent of PKA (Chen et al., 2000).
1.7 Adjuvants

Ramon originally described immunological adjuvants as ‘substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone’ (Ramon, 1924), but despite considerable research over many years, the only adjuvants currently approved for use with vaccines by the Federal Drug Administration of the USA are aluminium compounds (generically called alum) and the submicron squaline-water emulsion, MF59. Freund’s adjuvants, which are water-in-oil emulsions, have been extensively studied and are generally more potent than alum. Incomplete Freund’s adjuvant (contains oil and the emulsifier Arlacel) is not suitable for human use, whereas alum, which is used extensively, is a relatively poor adjuvant for many Ags and does not induce cell-mediated immunity (Gupta et al., 1993). Other adjuvants that have also been investigated include poly (D,L-lactide-co-glycolide) microparticles (PLG), monophosphoryl lipid A (MPL) and immune-stimulating complexes (ISCOMS), all with promising results (Ugozzoli et al., 1998). However, to date the most effective adjuvants, especially when delivered by the mucosal route, are AB-type bacterial toxins, including CT, *Escherichia coli* heat-labile enterotoxin (LT) and pertussis toxin (PT).

1.8 Cholera Toxin (CT)

1.8.1 Structure and function of CT

CT is a product of *Vibrio cholerae* and is the causative agent of the debilitating cholera diarrhoea (Spangler, 1992). This toxin is an extremely potent immunogen when administered by mucosal routes and also acts as a potent mucosal adjuvant, enhancing immune responses to coadministered Ags (Douce et al., 1997; Fontana et al., 1995). As a consequence, this molecule or derivatives thereof has been
extensively studied as a mucosal adjuvant for a wide variety of Ags in animal models and in a number of clinical trials.

CT is composed of two subunits, a monomeric A subunit and a pentameric B subunit. The B subunit (CTB) contains five identical polypeptide monomers that are arranged in a cylinder-like structure, with a central cavity that is responsible for binding to its eukaryotic cell receptor (Sixma et al., 1991). Interaction of CTB with its surface receptor is necessary for internalisation of the globular A subunit. The main receptor is GM1-ganglioside, \[\text{Gal(\(\beta\)1-3)GalNAc(\(\beta\)1-4)(NeuAc(\(\alpha\)2-3)Gal(\(\beta\)1-4)Glc(\(\beta\)1-1)ceramide}\], a glycosphingolipid, found ubiquitously on the cell surface of mammalian cells (Sixma et al., 1991).

The A subunit is linked to the B pentamer by a trypsin sensitive loop and a long \(\alpha\)-helix. The C-terminus of the \(\alpha\)-helix enters the central cavity of the B oligomer, thus anchoring the two subunits. The A subunit can be easily nicked by exogenous proteases between residues 192 and 195 to yield two fragments, \(A_1\) and \(A_2\), that remain linked by a disulphide bridge. The \(A_1\) fragment is responsible for the enzymatic activity associated with this toxin, while the \(A_2\) fragment represents an adaptor molecule, whose major function is in interacting with the B subunit. To become activated, the loop must be proteolytically cleaved and the disulphide bond reduced (Sixma et al., 1991).

After the toxin binds to the receptor via the B subunit, it is internalised and transported to the Golgi apparatus. Here the A and B subunits are disassembled and the A-subunit is transported to the endoplasmic recticulum (ER), whereas the B-subunit remains in the Golgi and is later degraded. The \(A_1\) subunit is then translocated from the ER to the cytosol, where it interacts with soluble ADP-ribosylation factors (ARFs), thereby becoming activated. Once activated, the \(A_1\)
subunit catalyses ADP-ribosylation of the $\alpha$ subunit of the GTP-binding protein that regulates the activity of adenylate cyclase ($G_{sa}$). ADP-ribosylation causes permanent activation of adenylate cyclase and abnormal intracellular accumulation of cAMP. Raised cAMP concentrations results in the phosphorylation of PKA and opens the cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ channels, resulting in the osmotic movement of water into the gut lumen and characteristic profuse watery diarrhoea (Rappuoli et al., 1999; Salmond et al., 2002; Williams et al., 1999) (Fig. 1.8). Although the major function of the $A_2$ fragment is in the interaction with the B subunit, the C-terminus also contains a sequence motif associated with retrieval of proteins from the trans-Golgi network to the ER (Pelham, 1992). It has been suggested that this be important for the delivery of $A_1$ fragment to the correct cellular compartment.

1.8.2 Adjuvant action of CT

CT is a powerful immunogen; low doses induce strong anti-toxin secretory and systemic antibody responses (Pizza et al., 2001; Rappuoli et al., 1999; Williams et al., 1999). CT also enhances immune responses against foreign bystander Ags. Co-administration of CT with Ag via the nasal, oral and other mucosal routes results in substantial enhancement of Ag-specific mucosal IgA and serum IgG responses. The adjuvant effects of CT have also been demonstrated in studies involving immunization via the subcutaneous, intraperitoneal, intravenous, intradermal and transcutaneous routes (Pizza et al., 2001; Rappuoli et al., 1999; Williams et al., 1999). In addition to enhancing humoral immunity, CT is also capable of augmenting cellular immune responses to coadministered Ags. The majority of studies examining the adjuvanticity of CT have demonstrated that this toxin enhances a Th2-biased
The intoxication process following infection with enterotoxigenic bacteria

The B subunit of the toxin facilitates entry into the cell by binding to GM1 receptors (a and b). The toxin is internalised and transported to the Golgi apparatus (c). Here the A subunit is cleaved and transported to the cytosol, via the endoplasmic reticulum (d). The toxin then ADP-ribosylates the α subunit of the GTP-binding protein (G_{so}) which regulates the activity of adenylate cyclase (e). Activation of adenylate cyclase results in the accumulation of excess cAMP (f) and activation of the cAMP-dependent signalling pathway, which involves protein kinase A (PKA) and the opening of chloride channels causing osmotic movement of water into the gut lumen.

Reproduced from Salmond et al., 2002.
response to bystander Ags, with elevated concentrations of Ag-specific T cell-associated IL-4, IL-5 and IL-10 production and IgG1 and IgE antibodies in immunized animals (Marinaro et al., 1995; Yamamoto et al., 1997a; Yamamoto et al., 1997b). However, mixed Th1/Th2 responses have also been demonstrated following oral immunization with CT and tetanus toxoid (Xu-Amano et al., 1994) or keyhole limpet hemocyanin (KLH) (Hornquist and Lycke, 1993) or following intranasal immunization with CT and fimbrial protein of Porphyromonas gingivalis (Yanagita et al., 1999). Furthermore, CT promoted a Th1-like response to the major birch pollen Ag (Wiedermann et al., 1998).

In addition to enhancing Th responses, CT can enhance Ag-specific class I restricted cytotoxic T lymphocyte (CTL) responses to coadministered Ag (Bowen et al., 1994; Simmons et al., 1999). CTL represent an important immune effector mechanism for clearance of many intracellular viruses and bacteria and may enhance the success of a vaccine against mucosally transmitted pathogens (Belyakov et al., 1998). Following recognition of processed viral and bacterial fragments presented on the infected cell surface, CTL destroy the infected cells. Unlike CD4^+ T cells, which recognize exogenous Ag in association with MHC class II, CD8^+ CTL recognize endogenous Ag associated with MHC class I molecules. Therefore, to generate an effective CTL response, an exogenous Ag has to be directed to the endogenous pathway of processing, for presentation to class I restricted T cells. Bowen et al. demonstrated that oral or intravenous administration of CT with the Ag OVA, primed mice for OVA-specific CTL (Bowen et al., 1994). An important role for DCs in the priming of CTL responses has been suggested. Progador et al. demonstrated that following co-immunization of OVA CTL peptide (OVA_{257-264}) with CT, only DCs from the nasal associated lymphoid tissue could present OVA_{257-264} to OVA-specific
T cells in vitro, suggesting that DCs may be the APC responsible for the presentation of OVA\textsubscript{257-264} in vivo (Porgador et al., 1998).

Initial studies indicated that the adjuvanticity of CT was solely dependent on its ADP-ribosyltransferase activity and subsequent accumulation of intracellular cAMP (Lycke et al., 1992), but more recently studies have shown that enzymatically inactive mutant toxins, which do not promote cAMP production, retain some mucosal adjuvant activity (Yamamoto et al., 1997a; Yamamoto et al., 1997b). The non-toxic CT mutants CTS61F and CTE112K, which are generated by site-directed mutagenesis, have some of adjuvant properties, with enhancement of Ag-specific IgG1 and IgE antibodies detected in mice immunized with Ag in the presence of these mutants (Ohmura et al., 2001; Yamamoto et al., 1997a; Yamamoto et al., 1997b). In addition, these mutant toxins enhanced Ag-specific CD4\(^+\) T cell proliferation and the Th2-associated cytokines IL-4, IL-5, IL-6 and IL-10, indicating that the adjuvant properties of CT may be independent of its enzymatic activity. Mutant toxins which retain residual enzyme activity e.g. CTS106 appear to be more potent adjuvants than non-toxic mutants, with similar properties to those of wild-type CT (Douce et al., 1997), indicating that the ADP-ribosylation activity does contribute to the adjuvant activity of this toxin.

The toxicity of CT has excluded its use as a mucosal adjuvant in humans. As a consequence CTB or CT mutants have been explored as an adjuvant for mucosal vaccinations in mice and in humans (Jertborn et al., 2001; Tochikubo et al., 1998). Initial descriptions of potent adjuvant properties of CTB later appeared to be due to contaminating active CT in the purified CTB preparations employed in these studies (Tamura et al., 1994). More recent studies with recombinant CTB have shown that it appears to have weak or variable adjuvant activity when compared with the holotoxin.
(Douce et al., 1997; Yamamoto et al., 1997a; Yamamoto et al., 1997b). Although CTB is a relatively ineffective adjuvant for admixed Ags, linking of certain Ags to CTB through chemical or genetic conjugation has been shown to be a highly efficient mechanism for the induction of mucosal or parental responses to the Ag (Toida et al., 1997). In addition, recombinant CTB can induce tolerance when conjugated with specific Ag. Coupling of CTB with myelin basic protein (MBP) suppressed the T-cell-mediated autoimmune disease EAE in the rat (Sun et al., 1996). Oral administration of CTB linked with insulin was also an effective therapy against diabetes in the nonobese diabetic (NOD) mice (Bergerot et al., 1997).

A novel immunomodulatory gene fusion protein, CTA1-DD, which combines the ADP-ribosylating ability of CT with a dimer of an Ig-binding fragment D of *Staphylococcus aureus* protein A has been developed (Ägren et al., 1997). This fusion protein is designed to specifically target B cells and acts as a potent mucosal adjuvant, enhancing IgG1 and IgG2a antibodies to coadministered Ags in mice (Ägren et al., 1997; Lycke and Schon, 2001), thus showing that the B subunit is not an essential component for the adjuvant action of CT.

The theory that the adjuvant action of CT is derived from an independent contribution of the A and B subunits might account for the wide diversity of results obtained using different subunits or mutants of CT. Furthermore, the route of administration, dose of toxin used, purity (LPS contamination was not ruled out in all studies) and nature and dose of the bystander Ag (McNeela and Mills, unpublished observations) may all influence the enhancement or suppression of Th1/Th2 responses.
1.8.3 Mechanism of adjuvant action

The mechanism of action of CT as a mucosal adjuvant has long been controversial and a number of hypotheses have been proposed to explain its activity. One mechanism by which CT can promote the immune response to a bystander Ag is through the enhancement of Ag uptake at the mucosal sites. When orally administered with dextran and KLH, CT increased intestinal permeability for dextran, in parallel with a strong enhancement of a KLH-specific immune response (Lycke et al., 1991). This ability of CT to facilitate access of luminal Ags to the mucosal immune system was dependent on the enzymatic activity of the toxin, as CTB failed to increase intestinal permeability or KLH-specific immune responses.

CT also acts as an adjuvant for parentally delivered Ag. The direct activation of cells of the immune system by CT may be a key component in the adjuvanticity of this toxin. As already discussed, induction of T cell subtypes is influenced by the cytokine profile and maturation status of DCs. CT induces the production of IL-1 from macrophages (Bromander et al., 1991) and enhances alloantigen presentation by cultured intestinal epithelial cells (Bromander et al., 1993). However, it has also been demonstrated that Ag processing can be inhibited by the ADP-ribosyltransferase activity of this AB bacterial toxin; CT but not CTB suppressed intracellular Ag processing in APCs (Matousek et al., 1998). In addition, CT can modulate the expression of co-stimulatory molecules on the surface of cells of the innate immune system. The expression of CD86 on bone marrow derived macrophages was enhanced by treatment with CT but not CTB (Yamamoto et al., 1999). Furthermore, CD86 expression by Mac1+ Peyer’s patch cells was increased after intraluminal exposure to CT. CT has also been shown to induce the upregulation of CD80 and
CD86 on murine Flt3 ligand-expanded DCs (Williamson et al., 1999) and on human DCs (Gagliardi et al., 2000).

Studies that have examined the effect of CT on cells of the immune system have shown the CT can activate Th2-inducing DCs through the selective inhibition of certain Th1-promoting factors and induction of Th2-associated factors (de Jong et al., 2002; Gagliardi et al., 2000; Lavelle et al., 2003). CT enhances LPS-stimulated IL-10, IL-6 and IL-1β production but decreased IL-12, TNF-α and nitric oxide (NO) production from macrophages (Cong et al., 2001). This increase in IL-10 and inhibition of IL-12 and TNF-α may be due to the increased production of cAMP induced by CT in these cells, as similar cytokine profiles were observed in LPS-treated macrophages in the presence of the cAMP elevating agent, 8-bromo-cAMP (Feng et al., 2000). CT also inhibited the production of a variety of pro-inflammatory cytokines and chemokines, including IL-12, TNF-α, MIP-1α and MIP-1β from DCs stimulated with LPS or CD40L (Gagliardi et al., 2000). These observations were mimicked by the cAMP-elevating agent forskolin, suggesting a role for the enzymatic activity of the toxin in this suppression. However, administration of CTB at the time of trinitrobenzene sulfonic acid (TNBS)-induced colitis (which is an IL-12 driven, Th1-cell-mediated colitis) inhibited the development of colitis (Boirivant et al., 2001). The administration of CTB was associated with the suppression of IFN-γ secretion, without an increase in IL-4 production and this inhibition of IFN-γ secretion was due to a marked inhibition of IL-12 production. Furthermore, pre-treatment of macrophages with CTB suppressed the induction of the pro-inflammatory cytokines TNF-α and IL-12p70, as well as NO synthesis induced by LPS (Burkart et al., 2002).

In addition to modulation of APCs, the adjuvant properties of CT may also involve the direct modulation of T cells. A recent study has shown the CTB can
directly enhance proliferation and cytokine production in Ag-primed CD4^ T cells (Wang et al., 2003), thereby indicating an additional mechanism whereby the toxin can promote immune responses to foreign Ags. Pre-treatment of APCs with CT or CTB can enhance IL-4 production by a T cell clone, by direct interaction of CTB-associated APCs and non-GM1 receptors on T cells (Li and Fox, 1996). Therefore, the modulation of immune responses by CT may be dependent on distinct contributions of the enzyme activity, the B subunit interaction with GM1-ganglioside, as well as other non-enzymatic activities of the A subunit and perhaps direct modulation of T cells.

1.9 Pertussis toxin (PT) – structure and function

PT is an AB bacterial endotoxin produced by the bacterial *Bordetella pertussis*, the etiologic agent of whooping cough and is one of its main virulence factors, playing a major role in its pathogenesis. The A monomer (S1) is responsible for its toxicity which is mediated by the ADP-ribosyltransferase activity associated with AB endotoxins, while the B oligomer mediates binding of the toxin to surface glycoproteins, including lactosylceramide and gangliosides and facilitates the entry of the A subunit into the cell. The pentameric B oligomer is composed of 4 subunits, S2 to S5, noncovalently linked, in a ratio of 1:1:2:1 (Kaslow and Burns, 1992; Tamura et al., 1982). PT differs from other AB endotoxins, including CT, in its cellular receptors and substrates (Spangler, 1992).

Like CT, PT possesses adjuvant properties, boosting immune responses to unrelated bystander Ags coadministered by systemic or nasal routes (Mu and Sewell, 1994; Munoz and Peacock, 1990; Roberts et al., 1995; Ryan et al., 1998). PT enhances both local and systemic antibody responses, including IgE (Munoz and
Peacock, 1990), IgG (Samore and Siber, 1996) and IgA (Roberts et al., 1995) to coadministered Ag. PT also enhances Ag-specific IFN-γ and IL-2, IL-4 and IL-5 production (Fischer et al., 2000; Sewell et al., 1986; Shive et al., 2000), induces delayed type hypersensitivity (DTH) responses (Tamura et al., 1985) and promotes certain organ-specific autoimmune diseases (Kamradt et al., 1991; Zou et al., 2000). In addition, PT, when co-injected with hen egg lysozyme (HEL) and incomplete Freund’s adjuvant (IFA), can switch the IFN-γ/IL-5+ Th2 response induced by HEL and IFA to a mixed Th1/Th2 response (Shive et al., 2000).

A number of studies have examined the effect of PT on innate cells, however, the levels of contamination endotoxin present in PT preparations was not determined in these studies and therefore the modulation of surface receptors and cytokines attributed to PT may involve synergy with LPS (Ryan et al., 1998; Shive et al., 2000). Indeed, recent studies have shown that PT synergises with LPS to enhance DC maturation and cytokine production (Ausiello et al., 2002) and promotes the generation of DCs that directs the development of a Th1 associated immune response (de Jong et al., 2002). Therefore, although PT is also an AB bacterial toxin, its effects on cells of the immune system are broader when compared with CT.
1.10 **Aims of this study**

- To confirm the adjuvant action of endotoxin-free CT and compare it with the immune response enhanced by PT
- To examine the affect of CT on the maturation of DCs and on the modulation of cytokine production from these cells
- To assess the role of the MAP kinase signal family in the modulatory effect of CT of DCs
- To examine the direct modulation of CT on T cells and assess the role of the MAP kinase cascade in this modulatory effect
2.1 Preparation of media and buffers

2.1.1 Cell culture medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 8% (v/v) heat inactivated (56°C for 30 mins) foetal calf serum (FCS; Lab Tech International), 100mM L-glutamine (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL; complete RPMI) was used for culture of all cells used in this work.

2.1.2 Phosphate-buffer-saline (PBS)

137 mM Sodium chloride (NaCl, 8 g)
8.1 mM Sodium hydrogen phosphate (Na$_2$HPO$_4$, 1.16 g)
1.5 mM Potassium dihydrogen phosphate (KH$_2$PO$_4$, 0.2 g)
2.7 mM Potassium chloride (KCl, 0.2 g)
Dissolved in 1 L of dH$_2$O and pH adjusted to 7.2 with hydrochloric acid (HCl)

2.1.3 Phosphate Citrate Buffer

0.5 M Citric Acid (10.19 g)
0.1 M di-sodium hydrogen orthophosphate 12-hydrate (Na$_2$HPO$_4$.12H$_2$O, 36.9 g)
Dissolved in 1 L of dH$_2$O and pH adjusted to 5 (NaOH)

2.1.4 FACS buffer

PBS pH 7.2
2% (v/v) FCS
0.1% (w/v) sodium azide (NaN$_3$)
2.1.5 Electrophoresis running buffer

25 mM Tris base (3 g)
200 mM glycine (14.4 g)
17 mM SDS (1 g)
dH$_2$O (1 L)

2.1.6 Transfer buffer

25 mM Tris-HCl pH 8.3 (2.73 g)
0.2 M Glycine (13 g)
20% (v/v) Methanol (180 ml)
0.05% (w/v) SDS (0.45 g)
dissolved in dH$_2$O (720 ml)

2.1.7 Strip buffer

50 mM Glycine-HCl pH 2.0
dH$_2$O

2.1.8 1 x sample buffer

62.5 mM Tris-HCl pH 6.8
10% (v/v) glycerol
2% (w/v) SDS
0.1% (w/v) bromophenol blue
dH$_2$O
50 mM DTT
2.1.9 TBE

10.78g Tris-HCl pH 8.3
5.5g Boric Acid
0.744g disodium EDTA dihydrogen

Dissolved in 1 L dH₂O

2.2 Bacterial toxins, Ags and inhibitors

CT was obtained from Sigma-Genosys (Poole, U.K.), PT was obtained from Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) and CTB was obtained from LIST Biological Laboratories (Campbell, CA). Depyrogenated KLH was purchased from CalBiochem (La Jolla, CA), phosphorothioate-stabilized CpG-ODN (5'-GCTAGACGTTAGCGT-3') was synthesized by Sigma-Genosys (Cambridge, U.K.) and \textit{E. coli} LPS (serotype 127:B8) was purchased from Alexis Biochemicals (UK Ltd.). All solutions were prepared in pyrogen-free water (Baxter, Maychem Sales, Dublin, Ireland) or Dulbecco’s PBS (Sigma-Genosys). The p38 inhibitor SB302580, the MEK inhibitors U0126 and PD98059 and the JNK inhibitor SP600125 were purchased from Alexis Biochemicals (UK Ltd.). The inhibitors were dissolved in dimethysulphoxide (DMSO; Sigma-Genosys) and stored at -20°C in the dark.

2.3 Removal of LPS from bacterial toxin preparations

LPS in the toxin preparations was removed on endotoxin removal columns (Detoxi-Gel endotoxin removing gel; Pierce, Rockford, IL) following manufacturers instructions. The columns were regenerated with a 1% solution of sodium deoxycholate in pyrogen-free water. The columns were then rinsed through with pyrogen-free water to remove the detergent and the sample was passed through using
gravity flow. The protein concentration of the endotoxin-free samples was
determined using the BCA assay and the sample was then UV sterilized (section 2.5
and 2.6).

2.4 LPS analysis

The level of LPS present in the toxin preparations was assessed using the
chromogenic *Limulus amebocyte* lysate (LAL) assay (BioWhittaker, Walkersville,
MD). Endotoxin levels were measured according to manufacturer’s instructions and
semi-quantified by a simple equation to give endotoxin amounts in endotoxin unit
(EU) per ml. CT and PT were highly contaminated with LPS (>1000 EU per mg of
protein) before purification. Following purification (section 2.3) endotoxin was
undetectable in the preparations. CTB contained < 1pg/ml of LPS per 400μg/ml of
protein and therefore removal of LPS was not required.

2.5 Protein quantification using the BCA assay

Protein concentration in toxin preparations was performed using the BCA assay kit
(Pierce, Rockford, IL) according to manufacturers instructions. Standards were
prepared from a stock solution of 2 mg/ml bovine serum albumin (BSA) in dH_{2}O.
Standards ranging from 31.25 μg/ml to 2000 μg/ml in a volume of 10 μl were added
to 96-well microtitre plates (Greiner bio-one, Germany) in triplicate. 10 μl of sample
was added to the plates in triplicate and 200 μl of the BCA solution was then added to
each well. The plates were incubated at 37°C for 30 mins to allow the reaction to
develop and the absorbance was measured at 562 nm using a microtitre plate reader
(Molecular Devices). The concentration of protein in the samples was then calculated
from the standard curve generated with the BSA standard.
2.6 UV-sterilization

Toxin preparations were sterilized by exposure to UV-irradiation at 354nm for 10 mins at a distance of 8 cm from the light source. Only preparations in medium free reagents were UV-irradiated, as the phenol red dye present in RPMI-1640 medium generated toxic free radicals upon exposure to UV light.

2.7 Animals

Specific pathogen-free female BALB/c (H-2d) mice were obtained from Harlan (Bicester, Oxon, UK) and were maintained in individually ventilated pathogen-free cages. The mice were bred and maintained under the guidelines of the European Union and the Irish Department of Health. All mice were 8-12 weeks old at the initiation of experiments.

2.8 Parental immunization of BALB/c mice

BALB/c mice were immunized either by subcutaneous (s.c.) injection into the footpad with a total volume of 50 µl or by intraperitoneal (i.p.) injection with a volume of 300 µl. Mice were immunized with PBS as control or with the Ag KLH (20 µg) in the presence or absence of the adjuvants CT (10 ng s.c. or 1 µg i.p.), PT (1 µg i.p.), LPS (20 µg s.c.) or CT plus LPS (10 ng and 20 µg respectively; s.c). Ag-specific immune responses were assessed 7 days after footpad and 14 days after i.p. immunization.

2.9 Detection of murine T cell responses in immunized mice

Immunized BALB/c mice were sacrificed by cervical dislocation one week after s.c. immunization or two weeks after i.p. immunization. Popliteal LN were aseptically
removed from s.c. immunized mice and pooled for each group of mice, or spleens were removed from i.p. immunized mice. Single cell suspensions were obtained by passing the cells through 70 μM cell strainers (Falcon, Becton Dickinson labware, Lincoln Park, NJ, USA), which were then washed and resuspended in fresh complete RPMI. Cells were stained with Trypan blue and viable cells were counted (section 2.10). Cells were adjusted to 2x10^6/ml in complete RPMI and cultured in triplicate wells of 96-well plates at 37°C and 5% CO2 with KLH (2-50 μg/ml) or with the mitogen phorpol myristic acetate (PMA; Sigma-Genosys; 25 ng/ml) and purified anti-mouse CD3 (α-CD3; BD PharMingen, SanDiego, CA; 1 μg/ml) or medium only as positive and negative controls respectively. Supernatants were collected after 24 hours, replaced with fresh medium and IL-2 protein concentrations were determined by enzyme linked immunosorbent assay (ELISA). After 72 hours of culture supernatants were removed and concentrations of IL-4, IL-5, IL-10, IL-13 and IFN-γ were determined by ELISA (see section 2.12).

2.10 Assessment of cell viability
Cell viability was determined using the dye Trypan blue (Sigma-Genosys), which is excluded from healthy cells but taken up by non-viable cells. Cells were counted using a haemocytometer and a bright light microscope.

2.11 Purification of T cells using a T cell purification kit
A mouse T cell CD4^+ subset column kit (R&D Systems, MN) was used, following manufacturers instructions, to purify CD4^+ T cells from the spleens of BALB/c mice via high affinity negative selection. Briefly, a single cell suspension of spleen cells was obtained by passing the cells through 70 μM cell strainers. The cells were then
depleted of erythrocytes by incubation in a sterile solution of 0.85% ammonium chloride (1 ml per spleen) for 5 min at 37°C. Cells were washed in column buffer provided in the kit and incubated with the contents of a 1 ml vial of monoclonal antibody cocktail also provided in the kit for 15 min at RT. The cells were washed thoroughly with column buffer and loaded onto the washed CD4 subset column. The cells were allowed to enter and then incubated in the column for 10 min at RT by closing the outlet value. The purified CD4⁺ T cells were then eluted from the column, washed in column buffer twice and resuspended in RPMI. The efficiency of the purification procedure was assessed by FACscan analysis using antibodies specific for CD3, CD4 and CD8 (section 2.16; Fig. 2.1).

The cells were stained with trypan blue, viable cells were counted (section 2.10) and adjusted to 1x10⁶ /ml. 96-well tissue culture plates were coated with anti-CD3 (10 μg/ml) in sterile PBS at 37°C for 4 hours. The wells were washed three times with sterile PBS to ensure excess antibody was removed. CD4⁺ T cells were plated in 200 μl volumes and incubated for 72 hours with or without addition of CT. 48 hours after stimulation supernatants were removed and analysed for cytokine production (section 2.12). Proliferation was determined after 72 hours of culture (section 2.11).

2.12 Determination of proliferative responses from T cells in vitro

Cells isolated from immunized mice and stimulated as outlined in section 2.9 were cultured for 4 days or CD4⁺ T cells purified and stimulated as outlined in section 2.11 were cultured for 72 hours. Cells were pulsed with 0.5 μCi of [³H]-thymidine (Amersham Pharmacia Biotech) in 25 μl of complete RPMI for the final 6 hours of culture. The cells were then harvested onto glass fibre filters (Wallac) with an automatic cell harvester. The filters were then dried and sealed in plastic sample bags.
Figure 2.1 FACs analysis demonstrating the purified of T cells isolated using a CD4+ subset column kit

CD4+ T cells were purified from the spleens of naïve BALB/c mice using the T cell CD4+ subset column kit and were stained with Abs specific for CD3, CD4 or CD8. Immunofluorescence is shown for (A) CD3+, (B) CD4+ or (C) CD8+ T cells (black histogram) compared with isotype control (grey histogram). The T cells purified were CD3+ and CD4+, but not CD8+.
(Wallac) with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac). $[^3]H$-thymidine incorporation was assessed using a Beta-plate scintillation counter (Wallac). Results are expressed as mean counts per minute (cpm) of $[^3]H$-thymidine incorporation for triplicate cultures.

2.13 Assessment of IL-2/IL-4/IL-5/IL-13/IFN-$\gamma$ concentrations by ELISA

The concentrations of IL-2, IL-4, IL-5, IL-13 and IFN-$\gamma$ were measured by ELISA using antibodies described in Table 2.1 and standards described in Table 2.2. Cytokine specific capture antibodies (50 $\mu$l/well at 1 $\mu$g/ml or 2.5 $\mu$g/ml for IL-13 in PBS) were added to high-binding 96-well microtitre plates and incubated overnight at 4°C. The plates were washed 4-5 times with wash buffer (PBS/0.05% Tween 20) and patted dry on paper towels as a final step. Non-specific binding sites were blocked with 150 $\mu$l/well of blocking solution (PBS supplemented with 5% w/v of non-fat dried milk) at RT for 2h. Following washing, plates were incubated overnight at 4°C with 50 $\mu$l/well of the test supernatant diluted 1 in 2 with medium or with the corresponding cytokine standard. The plates were then washed and incubated with 50 $\mu$l/well of biotinylated anti-cytokine antibody (1 $\mu$g/ml or 150 ng/ml for IL-13 in PBS) at RT for 1h. After washing, the plates were incubated for 20 mins at RT in the dark with 50 $\mu$l/well of horseradish-peroxidase (HRP) conjugated streptavidin (PharMingen, 1:1000 in PBS). After the final wash 100 $\mu$l of the substrate solution o-phenylenediamine (OPD) tablets (Sigma-Genosys) dissolved in phosphate-citrate buffer (section 2.1.3; 20 mg tablet in 50 ml of buffer plus 16 $\mu$l $H_2O_2$). The reaction was stopped with the addition of 50 $\mu$l of stop solution (1 M $H_2SO_4$). The O.D. value of test samples and cytokine standards were measured at 495 nm using a microtitre plate reader and cytokine concentrations for test samples were determined.
automatically by referral to a standard curve generated from recombinant cytokines of known concentration and potency using the SOFTmax Pro software. The limits for the assays were 30 pg/ml for IL-2, 40 pg/ml for IL-4 and IL-5, 200 pg/ml for IL-13 and 160 pg/ml for IFN-γ.

2.14 Quantification of KLH-specific IgG1 and IgG2a antibodies

Serum samples were prepared from peripheral blood removed from the thoracic cavity by aortic puncture of mice immediately after sacrifice by cervical dislocation. The concentrations of Ag-specific IgG1 or IgG2a antibodies in the sera of were determined by ELISA. KLH (5 μg/ml in PBS) was added to medium-binding 96-well microtitre plates and incubated overnight at 4°C. Excess Ag was washed off with wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by incubating the plates with PBS supplemented with 10% w/v of non-fat dried milk for two hours at RT. Serum was serially diluted in PBS on the washed plates to determine the endpoint titres. The initial dilution used was 1/50. The plates were then incubated overnight at 4°C. After washing, the plates were incubated for 1 hr at RT with 50 μl/well of alkaline phosphatase conjugated rat anti-mouse IgG1 monoclonal antibody (A85-1; PharMingen; 1:10,000 in PBS) or rat anti-mouse IgG2a monoclonal antibody (R19-15; PharMingen; 1:10,000 in PBS). The plates were then washed and incubated with 100 μl/well of OPD in phosphate-citrate buffer. The reaction was stopped with 1 M sulphuric acid and the absorbance was measured at 495 nm. Results are expressed as log_{10} endpoint titres determined by extrapolation to the OD 495 nm value of the control/naïve serum. Control wells in which Ag, serum sample or detection antibodies were omitted gave negligible absorbances with an O.D. range of 0.1-0.2.
2.15 Cell line culture

2.15.1 J558 -GM-CSF

The J558-GM-CSF cell line, a kind gift from Nathalie Winter (Institute Pasteur, Paris, France) is a GM-CSF expressing cell line. Cells were seeded at 1 x 10^5/ml in complete RPMI medium and maintained at 37°C in a humidity atmosphere of 5% CO_2. The cells were sub-cultured every 4-5 days and the supernatant was retained. After 10 passages the supernatants were pooled and the concentration of GM-CSF in the supernatant was determined by ELISA (section 2.17.1). The supernatant was diluted to 1 μg/ml of GM-CSF and used in culture at 4% (40 ng/ml).

2.15.2 EL4.II. -2 cells

The murine thymoma T cell line EL4.II.-2 was obtained from the America Type Culture Collection (ATTC) Rockville, USA and was cultured in complete RPMI medium at 37°C in a humidity atmosphere of 5% CO_2. For continuing cell culture the cells were seeded at 1 x 10^5/ml and sub-cultured every 3-4 days. For all assays the cells were passaged the day prior to use. For cytokine analysis the cells were typically seeded at 1 x10^6/ml in 96 well plates in 200 μl 12 hours prior to stimulation. Cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (BD PharMingen, SanDiego, CA; 10 μg/ml), PMA (as indicated) or PMA plus ionomycin (Sigma-Genosys; 1 μg/ml) for the indicated times. For western blot analysis the cells were typically seeded at 1 x10^6/ml in 6 well plates in 3 ml, 12 hours prior to stimulation. All experiments were carried out between passage number 6 and 10. After passage number 10 the cells were discarded.
2.15.3 Cryopreservation of cells in liquid nitrogen

Cells were frozen in complete RPMI medium containing 10% (v/v) DMSO and 20% FCS. The cells to be cryopreserved were first pelleted by centrifugation, resuspended with the freezing mix at 4°C and then transferred to cryo-tubes (Nunc) in 1ml volumes. Cells were frozen under conditions that permitted a gradual reduction of temperature (1°C/min) before transferral to liquid nitrogen. Recovery of viable cells from liquid nitrogen was performed by thawing the cells quickly in a 37°C water bath. The cells were then transferred to 10 ml centrifuge tubes and washed 3 times in cold RPMI-1640 to ensure that all traces of DMSO were removed from the cell suspension. A viability count was performed and cells resuspended in complete medium and cultured at 37°C in a CO₂ incubator as described above.

2.16 Isolation of bone marrow derived DCs

Bone marrow-derived DCs were generated from BALB/c (H-2^{d}) mice using a method similar to that described in Lutz et al. (1999). BALB/c mice were killed by cervical dislocation, their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with a 27-gauge needle and complete RPMI medium. A single cell suspension was obtained by passing the cells through 70 μM cell strainers. The resulting cells were washed, pelleted by centrifugation (1200 rpm for 5 mins) and resuspended in fresh complete RPMI medium. Immature DCs were prepared by culturing cells at 1x10^6/ml in complete RPMI medium supplemented with 4% supernatant from a J558-GM-CSF cell line (section 2.14.1). Three days later the supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh complete RPMI medium supplemented with 4% supernatant from the J558-GM-CSF cell line. The cells were
maintained for a further 4 days at 37°C with 5% CO₂. On day 7, the loosely adherent cells were removed and the viability of the DCs was assessed (section 2.10).

Cells were plated at 1 x 10⁶ /ml in 96-well plates in 200 μl for cytokine analysis, in 24-well plates in 1 ml for FACS analysis or in 6-well plates in 3 ml for western blot analysis 12 hours prior to stimulation.

2.17 Flow cytometry analysis

Surface marker expression on DCs was analysed by flow cytometry using a FACScan™ flow cytometer (Becton-Dickinson, San Jose, CA). The FACScan was calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads (Calibrate beads, Becton-Dickinson). DCs to be analysed were washed, resuspended in FACS buffer (section 2.1.14) and transferred to FACS tubes (Falcon). Cells were incubating with 50% FCS in PBS for 10 mins on ice to prevent non-specific binding. The cells were then stained with antibodies specific for CD80, CD86, CD40 or ICAM-1 and CD11c (Table 2.3) in FACs buffer for 30 mins on ice and washed three times in FACS buffer. Unstained cells were used as a control to determine the level of background auto-fluorescence, while cells stained with an irrelevant antibody of matched isotype was used to assess non-specific binding. The results were analysed using CELLQuest™ software. 30,000 cells were analysed per sample.

To determine the purity of CD4⁺ T cell (section 2.19), the above protocol was followed but the cells were stained with antibodies specific for CD3, CD4 or CD8 (Table 2.3).
2.18 Analysis of DC cytokine/chemokine secretion

2.18.1 Chemokine/cytokine detection using the DuoSet® ELISA development kits

The chemokines MIP-1α, MIP-1β, MIP-2 and MCP-1 and the cytokines IL-10, IL-12p70, TNF-α, IL-1β and GM-CSF concentrations were determined by ELISA using DuoSet® ELISA development kits (R&D Systems, MN) as recommended by the manufacturer. 50 μl/well of capture antibody (Table 2.4; in PBS) specific for the chemokine/cytokine in question was added to high-binding ELISA plates, which were incubated overnight at 4°C. After the wells were washed a minimum of 3 times with wash buffer (PBS/0.05% Tween 20) and patted dry on tissue paper, the plates were blocked with 200 μl/well of blocking solution (1% BSA, 5% sucrose in PBS) for 2 hours at RT to prevent non-specific binding. The plates were washed and 50 μl/well of the test supernatant diluted 1 in 2 with reagent diluent (1% BSA in PBS) or serially diluted chemokine/cytokine standards in reagent diluent were added and plates were incubated at 4°C overnight. After washing, 50 μl/well of biotinylated detection antibody (table 2.4; in reagent buffer) was added to each well and plates were incubated at RT for 2 hours. The plates were washed and incubated for 30 mins at RT in the dark with 50 μl/well of streptavidin-HRP (1:200 in reagent diluent). Finally after washing, the plates were incubated with 100 μl/well of OPD substrate in phosphate citrate buffer. The reaction was stopped with 50 μl of 1 M sulphuric acid. Absorbance was read at 492 nm and chemokine concentrations for test supernatants were determined by reference to a standard curve prepared for recombinant chemokines of known concentration and potency. The sensitivity limit of the assays was 30 pg/ml for each chemokine and for IL-12p70, TNF-α and IL-10 and 15 pg/ml for IL-1β and GM-CSF.
2.18.2 Detection of IL-12p40 by ELISA

IL-12p40 was detected by ELISA, using commercially available antibodies specific for the IL-12p40 subunits described in Table 2.1 and standard described in Table 2.2. High binding 96-well microtitre plates were coated overnight at 4°C with 50 µl/well (1 µg/ml in PBS) of purified anti-IL-12p40 monoclonal antibody. After washing in PBS/Tween, 150 µl/well of 5% dried milk/PBS was added to block non-specific binding sites and the plates were incubated at RT for 2 hours. Plates were washed and 50 µl/well of test supernatant diluted 1 in 500 in medium or serially diluted IL-12 standard was added and plates were incubated overnight at 4°C. The wells were washed and 50 µl/well of biotinylated anti-IL-12 antibody (1 µg/ml in PBS) was incubated for 1 hour at RT. IL-12p40 concentrations were detected by incubating washed plates with 50 µl/well of HRP-conjugated streptavidin (1:1000 in PBS) for 30 mins followed by washing and addition of 100 µl/well of the substrate solution OPD to the wells. The colour reaction was allowed to develop after which the reaction was stopped with the addition of 50 µl/well of 1 M H₂SO₄. The OD values were determined using a microtitre plate reader and cytokine concentrations for test samples were determined using a standard curve prepared using recombinant mouse IL-12 of known concentration and potency.

2.19 Western Blot Analysis

2.19.1 Preparation of samples for gel electrophoresis

Cells were seeded at a density of 1x10⁶ cells/ml, 3 ml per well in 6-well tissue culture plates. After appropriate stimulation, the supernatant was removed and the cells were lysed in 100 µl of 1 x sample buffer (2.1.8). Samples were then sonicated for 10 sec at 80% strength and boiled at 100°C for 5 mins.
2.19.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on Sodium Dodecylsulphate (SDS) polyacrylamide gel using a constant current on 30 mA per gel. Samples were first run through a stacking gel (1.3 ml 30% (w/v) acrylamide/bisacrylamide (Sigma-Genosys), 2.5 ml 1 M Tris-HCl pH 6.8, 60 µl 10% (w/v) ammonium persulphate (APS), 60 µl 10% (w/v) SDS and 10 µl TEMED made up to 6 ml with dH2O) and then resolved using a 12% polyacrylamide gel (6.3 ml 30% acrylamide/bisacrylamide, 3.75 ml 1.5 M Tris-HCl pH 8.8, 150 µl 10% APS, 150 µl 10% SDS, 6 µl TEMED made up to 15 ml with dH2O). Samples were run with pre-stained protein markers (Precision Plus Protein Standards; Bio-Rad) as molecular weight standards.

2.19.3 Transfer of proteins to membrane

The resolved proteins were transferred to nitrocellulose (Sigma-Aldrich) using a semi-dry transfer system. Briefly, the gel, nitrocellulose paper and filter paper were soaked in transfer buffer (2.1.6). The nitrocellulose paper was place on top of one piece of filter paper followed by the gel and then the other sheet of filter paper to make a layered sandwich. Air bubbles were then removed and the sandwich was placed on the anode of the semi-dry blotter, which has been pre-moistened with transfer buffer. The lid, containing the cathode was also pre-moistened and was firmly placed on top and the transfer was carried out at 225 mA for 75 mins.

2.19.4 Antibody blotting

Membranes were blocked to prevent non-specific binding by incubation in blocking buffer (5% (w/v) non-fat dried milk in 0.01% (v/v) PBS-Tween (PBS/T)) for 1h at RT. The membrane was rinse with wash buffer (0.01% (v/v) PBS/T) and incubated
with the relevant antibody at 1:200 to 1:2000 dilutions (Table 2.5) at 4°C overnight with constant movement. The membrane was washed for 5 mins in PBS/T five times at RT with constant motion. The membrane was then incubated with the appropriate secondary horseradish peroxidase (HRP) linked antibody as described in Table 2.5 for 1 hr at RT. Again the nitrocellulose was washed 5 times for 5 mins each with PBS/T. Blots were developed by SuperSignal® West Pico chemiluminescent substrate according to manufacturers instruction (Pierce, Rockford, IL). The membranes were placed between two sheets of transparent acetate before been exposed to photographic film for the required time. The film was processed using a Fuji X-ray processor.

2.20 Generation of T cell clones and lines

2.20.1 Preparation of APC

Whole spleens from naïve BALB/c mice were placed in vials containing ice-cold complete RPMI. These vials were inserted into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30 Gy, which prevents subsequent cell proliferation. A single cell suspension was generated as previously described and cell viability assessed.

2.20.2 Generation of LFH-specific and KLH-specific T cell lines

Dr. Miriam Brady generated T cell lines specific for liver fluke homogenate (LFH) as described below. Briefly, BALB/c mice were infected with the parasitic trematode *Fasciola hepatica* for 3 weeks. A single cell suspension of hepatic LN cells in complete RPMI medium was generated and stimulated with LFH (15 µg/ml) as Ag. After 7 days autologous irradiated spleen cells (APC, 2 x 10⁶ /ml; section 2.20.1) were added and 5 days later the cells were washed, centrifuged (1200 rpm for 5 min)
and resuspended at 1 \times 10^5 /ml with 5 U/ml of recombinant IL-2 (rIL-2). T cell lines were established by maintaining the cells at 1 \times 10^5 /ml with APC and Ag for 4-5 days followed by 7-8 day culture with rIL-2. Cells used in this study were cultured at 1 \times 10^5 /ml (200 \mu l per well in triplicate) with APC (1 \times 10^6 /ml) and Ag (LFH; 15 \mu g/ml) in the presence or absence of CT for 3 days.

T cell lines specific for the bystander Ag KLH were generated as described above from the spleen cells of mice isolated one week after immunized into the footpad with CT (10 ng) and KLH (20 \mu g). The same cycle of stimulation with APC and Ag (KLH 10 \mu g) followed by addition of rIL-2 (5 U/ml) 4 days later, followed 7-8 days later with APC and Ag as described above was used to maintain this T cell line. The cells were cultured at 1 \times 10^5 /ml (200 \mu l per well in triplicate) with APC (1 \times 10^6 /ml) and Ag (KLH; 10 \mu g/ml) in the presence or absence of CT for 3 days for this study.

2.20.3 Gene ration of FHA and HA specific T cell clones

Dr. Peter McGuirk generated the Th1 clone HDS2.6 specific for influenza virus HA and the Tr1 clone, FHA1.1, which is specific for FHA (Johnson et al., 2000; McGuirk et al., 2002). Briefly T cell lines were generated from T cells isolated from lung parenchyma and alveolar spaces from mice aerosol challenged with *Bordetella pertussis* or from spleen cells from mice immunized intra-muscularly with 50 \mu g haemagglutinin (HA) DNA from influenza virus as described above (section 2.20.2). After 2 rounds of Ag stimulation, the T cell lines were cloned by limiting dilution at 10, 5 and 1 cell(s)/well in 100 \mu l volumes of RPMI containing APC (2 \times 10^6 /ml), FHA (1 \mu g/ml) or influenza virus (10 HAU/ml) and rIL-2 (5 U/ml) in 96-well plates. Following 4-5 days incubation, rIL-2 was added to the cells to give a final
concentration of 5 U/ml, and 7-8 days later actively-growing T cell clones were
transferred in 1-2 ml volumes to 24-well plates and stimulated with APC, Ag and rIL-2. T cell clones were maintained by restimulation at the initial concentration of 1 x 10^5 /ml with Ag ad APC every 11-12 days, with rIL-2 added 4-5 days after stimulation, and were progressively expanded to 25 cm^2 tissue culture flasks. The specificity of the T cell clones were tested against FHA or influenza virus (10 HAU/ml) as previously described (McGuirk et al., 2002).

2.21 Determination of mRNA expression in EL4.IL-2 cells

2.21.1 RNA isolation from cells using TRI REAGENT™

EL4.IL-2 cells were cultured in 6 well plates, at 1x10^6 /ml and 3 ml per well with or without CT for the indicated times. Following treatment, cells were harvested for RNA extraction as follows: supernatants were removed, 1 ml of TRI REAGENT™ (Sigma-Genosys) was added per well, cells were scraped from the bottom of the plate using a syringe-barrel and nucleic acids were dissociated by repeated pipetting. Cells were transferred to a sterile, nuclease free 1.5 ml eppendorf and stored at -80°C until uses for RNA extraction. In order to extract total RNA, samples were allowed to reach room temperature and were then allowed to stand for a further 5 mins to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added to each tube, shaken vigorously for 15 sec and allowed to stand for 15 min at RT. Tubes were centrifuged at 13,000 rpm for 15 min at 4°C. The clear aqueous top layer containing the RNA was transferred to a fresh 1.5 ml eppendorf and 0.5 ml of isopropanol was added. The solution was mixed well and allowed to stand for 10 mins at RT. The resulting mixture was centrifuged for 10 mins at 13,000 rpm at 4°C. The supernatant was carefully removed, the RNA pellet was washed by adding 1 ml
of 75% ethanol in nuclease free water (Sigma-Genosys) and samples were centrifuged at 13,000 rpm for 10 min at 4°C. The ethanol was removed and any remaining ethanol was allowed to evaporate off. The RNA pellet was resuspended in 30-50 μl of RNase-free water and the RNA yield was calculated by reading the absorbance of the sample at 260 nm, using a spectrophotometer (Eppendorf) and applying the following formula:

Absorbance at 260 nm x 0.04 x dilution factor = μg RNA/μl sample

2.21.2 cDNA synthesis

First-strand cDNA was synthesised from 2 μg total RNA in a total reaction volume of 20 μl containing: 1 x reaction buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl, 3 mM MgCl₂), 1 mM of each dNTP (Bioline, London, UK), 1 μg oligo (dT) primer (Life Technologies Ltd, Paisley, UK), 10 U RNasin enzyme (Life Technologies Ltd.), and 100 U Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT; Life Technologies Ltd.). The reaction was incubated at RT for 10 min, 42°C for 60 min, followed by 10 min at 96°C to denature the reverse transcriptase, and cooled to 4°C for 10 min using a DNA engine DYAD, Peltier Thermal Cycler PCR machine.

2.21.3 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

For each 25 μl PCR reaction the following reagents are added: 2 μl of cDNA template, 2.5 μl of 10 x thermophilic buffer, 1.5 μl magnesium chloride (MgCl₂ 25 mM), 0.4 μl of dNTP (12.5 mM each; Promega), 0.5 μl of each of the appropriate forward and reverse primers (50 μM each; Table 2.6), 0.25 μl Taq polymerase (5 U/ml; Promega) and 17.35 μl of Dnase/RNase free water. The reaction takes place in a 0.2 ml PCR tube. The following program was used:
95°C for 5 min 1 cycle
94°C for 1 min
60°C for 1 min 35 cycles
72°C for 2 min
72°C for 10 min 1 cycle
4°C forever.

Exon-spanning primers were used to allow the discrimination of mRNA from genomic DNA contamination. Furthermore, to demonstrate the absence of genomic DNA contamination of RNA samples during subsequent reverse transcription reactions, RNAs was processed in parallel with MMLV-RT enzyme.

2.21.4 Analysis of PCR-products

PCR products were separated on 1% agarose gel (100 ml TBE buffer, 1 g agarose) in the presence of ethidium bromide (0.5 µg/ml) at 120 volts for 45 min. PCR product or DNA ladder (100bp; Promega) was mixed with blue/orange loading dye (Promega) before loading. Bands were visualised by UV irradiation using a gel doc system (Gene Genius Gel documentation and Analysis Systems, Syngene, Cambridge, UK).

2.22 Statistics

Statistical analysis of data was performed using the statistical package in the graphics software Prism. Cytokine and chemokine levels were compared by student t test if there were only two groups or otherwise by ANOVA. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify
differences between individual groups. \( p \) values of less than 0.05 are considered significant.
Table 2.1 The origin and specificity of antibodies used in ELISAs

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Clone/Reference</th>
<th>Conjugate</th>
<th>Supplier</th>
</tr>
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<tbody>
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<td>RA-6A2</td>
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Table 2.2 Origin of recombinant cytokines

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<td>IL-13</td>
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Table 2.3 The origin and specificities of antibodies used in FACs

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<th>Antibody Specificity</th>
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Table 2.4 The concentration of antibodies used from the DuoSet® ELISA kits

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<th>Detection antibody concentration</th>
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<td>IL-12p70</td>
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<td>MCP-1 (JE/CCL2)</td>
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<td>MIP-2</td>
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<td>GM-CSF</td>
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Table 2.5 The origin and specificities of antibodies used in western blotting

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<tr>
<th>Antibody Description</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>Primary Antibody Source</th>
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<tr>
<td>p-ERK (phospho-specific)</td>
<td>1/750</td>
<td>1/1500 HRP-linked goat anti-mouse IgG (Sigma-Genosys)</td>
<td>Santa Cruz Biotechnologies, USA</td>
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<td>E-4</td>
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<td>ERK1 (Total)</td>
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<td>1/4000 HRP-linked goat anti-rabbit IgG (Sigma-Genosys)</td>
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<td>K-23</td>
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<td>Phospho-p38 (Thr180/Tyr182)</td>
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<td>1/500 HRP-linked goat anti-rabbit IgG (Sigma-Genosys)</td>
<td>Cell Signal Technology, MA, USA</td>
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<td>p38 (total)</td>
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<td>1/4000 HRP-linked goat anti-rabbit IgG (Sigma-Genosys)</td>
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<td>I-15</td>
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<td>GATA-3</td>
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<td>HG3-31</td>
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<td>Monoclonal anti-β-actin</td>
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<td>AC-74</td>
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65
Table 2.6  Primer sequences used for PCR amplification

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<th>Mouse Gene</th>
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<td></td>
<td>Antisense: TAATCCAGGAACTGCCTCGT</td>
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<tr>
<td>GATA-3</td>
<td>Sense: TCTCACTCTCGAGGCAGCATGA</td>
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<td></td>
<td>Antisense: GGTACCATCTCGCCGCAACAG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: GGACTCTACGTCGGCGACGAGG</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Antisense: TCTTTGCCAATAGTGATGACCTTGGC</td>
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Chapter Three

Role of CT as an immunomodulator *in vivo* and *in vitro*
Chapter 3

3.1 Introduction

CT is a potent vaccine adjuvant when administered via the mucosal, subcutaneous or transcutaneous routes, enhancing antibody and T cell responses to coadministered Ags, as well as inducing antitoxin antibody and T cell responses (Williams et al., 1999; Xu-Amano et al., 1993). The majority of studies examining the adjuvant action of CT conclude that the toxin promotes a Th2-biased response to itself and coadministered Ags (Marinaro et al., 1995; Wilson et al., 1991; Xu-Amano et al., 1993). These responses are characterised by a dominant IgG1 serum antibody response (Marinaro et al., 1995; Vajdy and Lycke, 1992; Yamamoto et al., 1997a), as well as CD4+ T cell production of the Th2-associated cytokines IL-4, IL-5, IL-6 and IL-10 (Okahashi et al., 1996; Yamamoto et al., 1997a). However, enhanced Ag-specific IFN-γ and IgG2a antibody titres have also been reported with CT in vivo (Jones et al., 2001).

AB bacterial toxins function as adjuvants by activating both innate and adaptive immune responses through direct interactions with APCs and lymphocytes, suggesting that the adjuvanticity of CT may be a complex multistep phenomenon resulting from the interaction of this toxin with different cells of the immune system. However, the exact mechanism of adjuvant action of CT, as well as other AB bacterial toxins, has long been controversial and still remains unclear.

The effect of CT on cells of the innate immune system has been examined in some detail. CT has been shown to induce the production of IL-1α from macrophages (Bromander et al., 1991) and enhance alloantigen presentation by cultured intestinal epithelial cells (Bromander et al., 1993). This toxin also enhanced the expression of the costimulatory molecule CD86 on murine macrophages in vitro (Yamamoto et al.,
1999), while other studies have shown an increase in both CD80 and CD86 expression on B cells (Ågren et al., 1997). A role for CD86 in the adjuvant action of CT was demonstrated with the use of neutralizing antibodies to this costimulatory molecule; anti-CD86 inhibited KLH-specific serum IgG and mucosal IgA responses following oral administration of KLH and CT (Cong et al., 1997). CT can also inhibit IL-12p70 production from human monocyte-derived DCs in a dose dependent manner (Braun et al., 1999), thereby illustrating a potential mechanism employed by the toxin to suppress Th1 cell development.

While there is evidence that CT can mediate its effects through costimulation of surface molecules and cytokine production, the intracellular signalling pathways involved in the polarisation of the immune response by CT are not completely understood. In addition, previous results may be compromised due to the possible presence of contaminating endotoxin in the CT preparations used in these studies. Therefore, the aim of this investigation is to elucidate the mechanism of adjuvant action of CT (devoid of all LPS) and examine the role of the important signalling cascade, the MAP kinase signalling family, in this action. Initial studies focused on defining the adjuvant action of endotoxin-free CT compared to another AB bacterial toxin, pertussis toxin (PT), and subsequently, the in vitro modulation of DCs by CT. In addition, the ability of CT to modulate a TLR-ligand-induced T cell response in vivo was assessed.

In this chapter I confirm that CT is a potent adjuvant, inducing a local Th2/Tr1 response to a coadministered Ag and demonstrate that the immune response induced is more polarised than that generated by PT, which induces a mixed Th cell response. This confirms other studies which have separately examined the adjuvant properties of these toxins (Lycke, 1997; Ryan et al., 1998). The data also shows that CT directly
activates DCs by upregulating CD80 and CD86 expression and inducing MIP-2 production, in addition to modulating TLR-ligand-induced cytokine production. The data presented here also provides evidence that CT and the TLR4-ligand *E. coli* LPS induce different classes of CD4<sup>+</sup> T cell responses *in vivo*, which can counter-regulate each other.
3.2 Results

3.2.1 CT induces a Th2-specific immune response to a coadministered Ag in vivo

The adjuvant and immunomodulatory properties of CT were determined by examining the ability of CT to enhance T cell responses to KLH administered by a parental route. BALB/c mice were immunized subcutaneously (s.c.) in the footpad with PBS, KLH alone or with KLH plus CT, the draining popliteal LN were excised 7 days later and a single cell suspension was prepared. No response to Ag stimulation in vitro could be detected in the LN cells of control mice immunized with PBS (Fig. 3.1). Cells from mice immunized with KLH alone proliferated and secreted low levels of IL-4 and IL-10 when stimulated with Ag in vitro, which were not significant compared to medium control. Compared with KLH alone, immunization with KLH in the presence of CT significantly enhanced IL-4, IL-5, IL-10 and IL-13 production, which was associated with a significant increase in Ag-specific proliferation. Restimulation of these cells in vitro with Ag induced a modest increase in IFN-γ production, which was significant at only one of the doses of Ag used (Fig. 3.1). These results illustrate that CT induces a predominantly Th2 response to coadministered Ag in vivo.

The adjuvant action of CT was then compared to PT. BALB/c mice were immunized by the intraperitoneal route (i.p.) with KLH or with KLH in the presence of PT or CT. The immune response was examined in spleen cells 14 day later by stimulation with Ag in vitro. Spleen cells from control mice, which were immunized with PBS, and mice immunized with KLH alone did not produce any of the cytokines measured when stimulated in vitro with Ag (Fig. 3.2). In contrast, a potent Ag-specific response was observed in spleen cells from mice immunized with KLH in the
Female BALB/c mice (n = 5) were immunized s.c. in the footpad with PBS, KLH (20 μg) or KLH (20 μg) plus CT (10 ng). Popliteal LN cells, isolated 7 days later, were stimulated *in vitro* with KLH (2-50 μg/ml) or medium alone. Supernatants were removed after 3 days and were tested for IL-4, IL-5, IL-10, IL-13 and IFN-γ by ELISA. Supernatants were replaced with medium, ^3^H-thymidine was added, and cells were incubated at 37°C for 6h. Cells were harvested, and proliferation was determined by measuring thymidine incorporation. Results represent the mean (± SD) of five mice per group. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (KLH vs CT + KLH).
Figure 3.2 PT enhances Th1 and Th2 responses, while CT enhances a Th2/Tr1 response to co-administered Ag in vivo

Female BALB/c mice (n = 5) were immunized intraperitoneally (i.p.) with PBS, KLH (20 µg), KLH plus PT (1 µg) or KLH plus CT (1 µg). Spleen cells, isolated 14 days later, were stimulated with KLH (2 – 50 µg/ml) or medium alone. Supernatants were collected after 24 hours and were tested for IL-2. 3 days later additional supernatants were removed for analysis of IL-4, IL-5, IL-10 and IFN-γ by ELISA and replaced with fresh medium. 3H-thymidine was added, and cells were incubated at 37°C for 6h. Cells were harvested, and proliferation was determined by measuring thymidine incorporation. Results represent the mean (±SD) of five mice per group and are representative of two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (KLH vs PT + KLH).
presence of PT, with high levels of both the Th1-associated cytokines IL-2 and IFN-\(\gamma\) and the Th2-associated cytokines IL-4, IL-5 and IL-10 detected (Fig. 3.2). Enhanced proliferation was also observed in spleen cells from mice immunized with KLH plus PT when compared to mice immunized with KLH alone. This cytokine profile indicates that PT induces a mixed Th1/Th2 systemic response to coadministered Ag. In contrast, spleen cells from mice immunized with KLH plus CT did not produce detectable levels of IL-2, IL-4 or IFN-\(\gamma\) when stimulated \textit{in vitro} with Ag. The cells did however produce detectable levels of the Th2/Tr1-associated cytokines IL-5 and IL-10 in response to antigenic stimulation and proliferation was also enhanced when compared with cells from mice immunized with KLH alone (Fig. 3.2).

An examination of the IgG subclasses in the serum of immunized mice was consistent with the cytokine profiles (Fig. 3.3). Mice immunized with KLH plus PT and KLH plus CT had significantly higher levels of both IgG1 and IgG2a subclasses when compared with mice immunized with KLH alone, indicating that both toxins are potent enhancers of antibody responses. However, mice that were immunized with KLH plus CT had significantly lower levels of the Th1-associated antibody subclass IgG2a than those detected in the serum of mice immunized with KLH plus PT.

In summary, PT induces a mixed Th1/Th2 immune response to coadministered Ag, as illustrated by enhanced IL-2, IL-4, IL-5, IL-10 and IFN-\(\gamma\) production from stimulated spleen cells, as well as elevated serum IgG1 and IgG2a antibody titres. In contrast, CT induces a predominantly Th2/Tr1 response to systemic immunization with Ag \textit{in vivo}, with elevated IL-5 and IL-10 concentrations in the absence of IL-2, IL-4 and IFN-\(\gamma\), in addition to a predominant enhancement (compared to PT) of serum IgG1 antibody titres.
Figure 3.3 PT and CT enhance Ag-specific antibody (Ab) responses in vivo
Female BALB/c mice (n = 5) were immunized intraperitoneally (i.p.) with PBS, KLH (20 μg), KLH plus PT (1 μg) or KLH plus CT (1 μg). Serum was removed from the mice on day 14 and KLH-specific IgG1 and IgG2a antibody titres were determined by ELISA. Results are the mean (±SD) log_{10} antibody end-point titres for five mice per group. ***, p < 0.001 (KLH vs toxin + KLH); ++++ P < 0.001 (PT + KLH vs CT + KLH).
3.2.2 Modulation of DC responses by CT in vitro

Polarization of a T cell response in vivo is influenced by the APC and the cytokine milieu at the time of the T cell differentiation process. Since DCs are considered the primary APC, the effect of CT on bone marrow-derived DCs was assessed by examining the ability of this toxin to induce maturation and activation of these cells and to modulate the cytokine profile induced by known TLR-ligands.

3.2.2.1 Effect of CT on maturation of DCs

To assess if the action of CT is at least in part mediated through maturation of DCs, the effect of CT on the surface marker expression of immature DCs was assessed and compared to the surface marker expression following stimulation with the potent maturation stimulus LFS. Immature DCs are characterised by the low expression of the co-stimulatory markers CD80 and CD86 (grey histograms, Fig. 3.4). Incubation of DCs with LFS resulted in an increase in CD80, CD86, ICAM-1 and CD40 expression on the surface of these cells (black line) as compared to cells incubated with medium alone (grey histogram). Stimulation of immature DCs with CT resulted in a clear upregulation of CD80 and CD86 on the cell surface (left column, Fig. 3.4), but had little effect on ICAM-1 and CD40 expression in comparison to control cells. Therefore, both CT and LFS enhance the expression of the co-stimulatory molecules CD80 and CD86 but CT differs from LFS in its inability to upregulate the Th1-associated surface molecules CD40 and ICAM-1.

The ability of CT to modulate LFS-induced costimulatory molecule upregulation was also examined. Co-incubation of DCs with CT plus LPS resulted in a further increase in LFS-induced CD80 expression (mean fluorescence intensity (MFI) LFS = 15.06; LPS + CT = 24.37; Fig. 3.4). Surprisingly, this co-incubation
Immature DCs were stimulated with medium alone, CT (1 μg/ml), LPS (10 ng/ml) or CT plus LPS. After 12h incubation, cells were washed and stained with antibodies specific for CD80, CD86, ICAM-1 and CD40 or with isotype control antibodies. Immunofluorescence is shown for treated (black line) compared with untreated (gray histograms) DCs. The numbers on the right of each histogram refer to the MFI of the treated cells; the value for cells treated with medium alone is presented on the left of the first histogram in each case. Profiles are shown for a single experiment and are representative of three experiments.
resulted in the decrease of CT induced CD80 expression (MFI CT = 32.42; LPS + CT = 24.37). CD86 expression was slightly reduced in the presence of LPS and CT compared with that of cells treated with LPS only. CT marginally decreased LPS-induced ICAM-1 expression; however, CT suppressed LPS-driven CD40 expression on DCs, reducing the MFI from 17.55 to 8.80, indicating that CT can negatively regulate the expression of the Th1-associated co-stimulatory molecule CD40.

3.2.2.2 Modulation of chemokine production by CT

Since CT induces DC maturation by upregulating CD80 and CD86, the ability of CT to induce chemokine production from these cells was assessed. LPS, which was used as a control, induces the expression of a number of chemokines, including the CC chemokines MIP-1α, MIP-1β, MCP-1 and the CXC chemokine MIP-2 (Fig. 3.5). In contrast, exposure of DCs to CT elicited the production of MIP-2, but had no effect on MIP-1α, MIP-1β or MCP-1 protein concentrations.

3.2.2.3 Effect of CT on cytokine production

Stimulation of DCs with LPS induced the production of IL-10, IL-1β, TNF-α, IL-12p70 and IL-12p40 (Fig. 3.6). In contrast, incubation with CT alone did not induce the expression of any of these cytokines. However, CT did modulate LPS-induced cytokine production from DCs. Co-incubation of CT with LPS resulted in the significant augmentation of IL-10 and IL-1β production compared with that observed with LPS alone. In contrast, CT significantly suppressed the production of TNF-α, IL-12p70 and IL-12p40 (Fig. 3.6).

Although CT alone could not stimulate IL-10 production, CT synergised with an additional microbial stimulus to induce this cytokine (Fig. 3.7). This synergy with
Figure 3.5 CT stimulates MIP-2 but not MIP-1α, MIP-1β or MCP-1 production from DCs in vitro

Immature DCs were incubated for 15 hours with medium, CT (1 µg/ml) or LPS (10 ng/ml) as a positive control. Chemokine concentrations were determined in supernatants by ELISA. Results are the mean (± SD) of triplicate assays and are representative of four independent experiments. ***, p < 0.001 (untreated vs LPS); ###, p < 0.001 (untreated vs CT).
Figure 3.6 CT synergises with LPS to enhance IL-10 and IL-1β production, while inhibiting TNF-α, IL-12p70 and IL-12p40

Immature DCs were incubated with or without LPS (1 ng/ml for IL-10, IL-12p40, TNF-α, IL-1β or 100 ng/ml for IL-12p70), in the presence of CT (100 ng/ml; + CT) or absence (- CT) for 6 hours. Cytokine concentrations were determined in supernatants by ELISA. Results are the mean (± SD) of triplicate assays and are representative of four independent experiments. ***, p < 0.001 (with vs without CT).
LPS was evident as early as six hours after stimulation and continued for at least 26 hours (Fig. 3.7A). CT not only synergised with the TLR4-ligand LPS but also with the TLR9-ligand CpG, to enhance IL-10 production (Fig. 3.7B). 10 ng/ml of LPS was sufficient to induced low levels of IL-10 from DCs, however co-incubation with CT significantly augmented this (Fig. 3.7C). This synergy was observed with increasing doses of LPS up to at least 1000 ng/ml.

The inhibitory effect of CT on the production of some pro-inflammatory cytokines was determined in response to CpG as well as LPS. LPS and CpG are known inducers of the inflammatory cytokines IL-12p70 and TNF-α (Fig. 3.8), with both stimulating high levels of these cytokines after 6 hours. Co-incubation of either of these ligands with CT resulted in the significant suppression of both IL-12p70 and TNF-α.

Since the immune response induced by CT differed considerably from that induced by PT in vivo (Fig. 3.2) the effect of these two AB bacterial toxins on cytokine production from DCs was compared. PT, like CT, did not induce IL-12p70 or IL-10 production directly from these cells (Fig. 3.9). Co-incubation with PT significantly enhanced LPS-induced IL-12p70 production (Fig. 3.9A), while in contrast CT significantly inhibited the production of this inflammatory cytokine. In addition, PT reduced LPS-induced IL-10 production while the presence of CT resulted in a significant enhancement of this anti-inflammatory cytokine.

3.2.3 CT suppresses LPS-induced Th1 cell development in vivo

As discussed in section 3.2.1, CT induces a Th2-type response to coadministered Ag in vivo. However, due to the ability of CT to significantly enhance LPS-induced IL-1β and IL-10 production and to suppress LPS-induced IL-12 and
Figure 3.7 CT synergises with LPS or CpG to enhance IL-10 production

(A) Immature DCs were incubated for 6 - 26 hours with CT (1 μg/ml), LPS (10 ng/ml) or CT plus LPS. (B) Immature DCs were incubated with LPS (1 μg/ml) or CpG (10 μg/ml) in the presence or absence of 1 μg/ml of CT for 6 hours. (C) Immature DCs were incubated for 6 hours with increasing doses of LPS (10-1000 ng/ml) alone (-CT) or in the presence (+CT) of CT (1 μg/ml). Supernatants were analysed for IL-10 by ELISA. The results are the mean (± SD) of triplicate assays and are each representative of three independent experiments. ***, p < 0.001 (with vs without CT).
Figure 3.8 CT inhibits LPS- or CpG-induced IL-12p70 and TNF-α production

Immature DCs were incubated with medium, CT (1 µg/ml), LPS (1 µg/ml), LPS + CT, CpG (10 µg/ml) or CpG + CT for 6 hours. IL-12p70 and TNF-α concentrations were determined in the supernatants by ELISA. Results are the mean (± SD) of triplicate assays and are representative of three independent experiments. ***, p < 0.001 (with vs without CT).
Figure 3.9 CT and PT have opposing effects on LPS-induced IL-10 and IL-12p70 production

Immature DCs were stimulated with medium, PT (1 μg/ml) or CT (1 μg/ml) for 1 hour prior to the addition of LPS (1 μg/ml). IL-10 concentration was assessed after 6 hours and IL-12p70 concentration was determined after 24 hours by ELISA. Results are the mean (± SD) of triplicate cultures and represents three experiments. *, p < 0.05; ***, p < 0.001 (with vs without toxin).
TNF-α *in vitro*, we postulated that this modulation might occur *in vivo*. To address this hypothesis, the immune responses induced by CT and LPS alone or combined was assessed.

Mice were immunized in the footpad with KLH, KLH and CT, KLH and LPS or KLH and CT and LPS. Single cell suspensions of cells from the popliteal LN were prepared and stimulated with various concentrations of Ag (KLH) *in vitro*. When compared to cells from mice immunized with KLH alone, Ag-stimulation of cells from mice immunized with CT and KLH secreted high levels of the Th2-associated cytokines IL-4, IL-5, IL-13 and IL-10, with modest levels of IFN-γ *in vitro* (Fig. 3.10). Immunization of mice with KLH plus LPS resulted in the significant enhancement of Ag-specific IL-10, IFN-γ and T cell proliferation. Ag-stimulation of these cells *in vitro* did not, however, induce significant levels of the Th2-associated cytokines IL-4, IL-5 or IL-13. Thus, LPS enhanced a Th1 and Tr1 response to coadministered Ag *in vivo*, with high levels of IFN-γ and IL-10 with little IL-4, IL-5 or IL-13.

Co-administration of KLH and CT and LPS resulted in an immune response which was significantly distinct to that induced by KLH and LPS only (significant differences are marked with # in Fig. 3.10). Although LPS did not promote significant levels of Ag-specific IL-4 or IL-13 when administered *in vivo*, co-administration of CT with this TLR ligand resulted in a significant increase in these cytokines. An increase in IL-5 was also observed from these cells, although it did not reach significance. In addition, the levels of Ag-specific IL-10 were significantly higher in cells from mice immunized with KLH and CT and LPS, compared with mice that received KLH and LPS only. In contrast, immunization with KLH and CT and LPS *in vivo* induced lower levels of Ag-specific IFN-γ compared with mice that
Figure 3.10 CT can modulate LPS-induced responses to coadministered Ag in vivo. Female BALB/c mice (n = 5) were immunized s.c. in the footpad with PBS, KLH (20 μg), KLH and CT (10 ng), KLH and LPS (20 μg) or KLH and CT and LPS. Popliteal LN cells, isolated 7 days later, were stimulated in vitro with KLH (2-50 μg/ml) or medium alone. Supernatants were removed after 3 days and tested for IL-4, IL-5, IL-10, IL-13 and IFN-γ by ELISA. Supernatants were replaced with medium, 3H-thymidine was added, and cells were incubated at 37°C for 6h. Cells were harvested, and proliferation was determined by measuring thymidine incorporation. Results represent the mean (±SD) of five mice per group. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (KLH vs LPS + KLH). +, p < 0.05; ++, p < 0.01; +++, p < 0.001 (CT + KLH vs CT + LPS + KLH). # p < 0.05; ##, p < 0.01; ###, p < 0.001 (LPS + KLH vs CT + LPS + KLH).
received KLH and LPS only. In addition, administration of KLH and CT and LPS resulted in a modest but significant reduction in Ag-specific proliferation when compared to mice immunized with KLH and LPS only. Therefore, the presence of CT suppresses the Ag-specific Th1-biased response induced by LPS \textit{in vivo} and shifts it towards a Th2-like response.

The levels of Ag-specific IL-4, IL-5, IL-10 and IL-13 produced by mice that received KLH and CT and LPS were significantly lower than those from mice immunized with KLH and CT only (significant differences are marked by + in Fig. 3.10), while IFN-γ was significantly increased.

Analysis of the Ag-specific serum IgG antibody subclasses also supports these observations; CT induced slightly more IgG1 than IgG2a, while LPS preferentially enhanced IgG2a (Fig. 3.11). When both CT and LPS were coadministered \textit{in vivo}, similar levels of Ag-specific IgG1 and IgG2a were detected.

In summary, CT induced a Th2 response \textit{in vivo} while LPS induced a Th1 type response. Immunization with LPS and CT plus KLH resulted in the increase in LPS-induced IL-4, IL-5 and IL-13, with significant enhancement of IL-10 production and inhibition of the Th1-associated cytokine IFN-γ. In contrast, CT-induced Th2 cytokines were reduced and IFN-γ was enhanced by the co-administration of CT and LPS plus KLH \textit{in vivo}. Therefore, not only can CT and LPS individually regulate the Th cell response \textit{in vivo}, these microbial molecules can switch the Th1/Th2 response induced by the other towards the opposite phenotype.
Figure 3.11 Addition of LPS augments IgG2a responses with CT in vivo

Mice were immunized as described in the legend of figure 3.10. Serum was removed 7 days later and KLH-specific IgG1 and IgG2a antibody titres were determined by ELISA. Results are the mean (±SD) of log_{10} antibody end-point titres for five mice per group. *, p < 0.05 (KLH vs CT + LPS + KLH); #, p < 0.05 (CT + KLH vs CT + LPS + KLH).
3.3 Discussion

One of the principal features of the adaptive immune system is its ability to generate diverse types of immunity in response to numerous pathogens. These responses vary with respect to the cytokines secreted by Th cells and the class of antibody produced by B cells. Adjuvants are used to boost both T and B cell responses to numerous Ags. The use of microbial toxins as adjuvants has been under investigation for a number of years. CT, as well as a number of other AB toxins, has been shown to be a potent immune activator, enhancing T cell and antibody responses to coadministered Ags. Numerous reports, including data presented here, indicate that CT promotes a strong Th2-biased response, with high levels of Ag-specific IL-4, IL-5, IL-10 and IL-13 with little or no IFN-γ (Fig. 3.1) (Marinaro et al., 1995; Xu-Amano et al., 1993; Yamamoto et al., 1997a). The selective enhancement of Th2 responses is consistent with the antibody classes enhanced, with IgE (Marinaro et al., 1995; Simecka et al., 2000; Yamamoto et al., 1997a) and higher titres of IgG1 than IgG2a (Douce et al., 1997; Ruedl et al., 1996) detected after immunization with Ag in the presence of CT. In contrast, another AB bacterial toxin, PT, induces a mixed Th1/Th2 type response, with enhanced Ag-specific IL-2, IL-4, IL-5, IL-10 and IFN-γ (Fig. 3.2) which is consistent with previous studies (Mu and Sewell, 1993; Roberts et al., 1995; Ryan et al., 1998). Furthermore, the antibody subclasses induced in the serum of immunized mice mirror these T cell responses, with CT and PT enhancing both IgG1 and IgG2a antibody titres, but with CT inducing significantly less IgG2a compared with PT (Fig. 3.3).

Although it appears that CT selectively enhances Th2 responses, our group has shown that it may also enhance Tr1 type responses. An assessment of the IL-10 producing capacity of T cells from the spleens of mice immunized with Ag in the
presence of CT showed that these cells, when cloned by limiting dilution, contain a subset of Ag-specific Tr cells, which produce IL-10 and IL-5, with little or no IFN-γ or IL-4 (Lavelle et al., 2004; Lavelle et al., 2003). The cytokine profile of Ag-stimulated spleen cells from mice immunized with KLH and CT shown in Fig. 3.2 is consistent with this conclusion and is in sharp contrast to the immune response induced by PT. Therefore, although CT and PT are both AB bacterial toxins and are potent adjuvants, they promote contrasting T cell responses and consequently can be used as powerful tools to manipulate the immune system and further our understanding of the regulation of Th cell responses.

The mechanisms employed by these toxins to selectively enhance distinct T cell subclasses in vivo may be a complex phenomenon, resulting from the interaction of these toxins with a variety of immune cells. DCs are now emerging as pivotal cells in this process (Banchereau et al., 2000; Kalinski et al., 1999; Moser and Murphy, 2000). The ability of a DC to initiate a specific T cell response has been under much debate over the last number of years, with suggestions that the origin, state of maturation and dose and type of Ag all play a role (discussed in section 1.3.1 and 1.3.2). In addition, pathogen-derived products and/or inflammatory mediators have been shown to influence the ability of DCs to direct Th1, Th2 or Tr1 cell development (de Jong et al., 2002; McGuirk et al., 2002). Using an adoptive transfer technique, we have shown that CT-stimulated DCs pulsed with Ag can induce Ag-specific Th2 and Tr1 cells in recipient mice, characterised by the predominant production of Ag-specific IL-10 (Lavelle et al., 2003).

Data presented here demonstrates that CT, in the absence of any other exogenous stimuli, can upregulate cell surface expression of the co-stimulatory molecules CD80 and CD86 on DCs, which is consistent with the findings from other
groups (Cong et al., 1997; de Jong et al., 2002; Gagliardi et al., 2000). However, the present study also demonstrates that CT can reduce LPS-induced CD40 expression. We have previously shown that CT upregulates OX40 expression, but does not affect the levels of MHC class II expressed on the surface of DCs (Lavelle et al., 2003). Costimulatory molecules play an important role in the efficient interaction between DCs and T cells and therefore the initiation of the adaptive immune response. In particular MHC class II, CD80 and CD86 have been shown to be critical for the initiation of the primary immune response (Lespagnard et al., 1998) and also contribute to the polarisation of Th1 or Th2 subsets (Kuchroo et al., 1995). Indeed, the upregulation of CD86 expression on macrophages was implicated in the selective enhancement of Th2-type responses by CT in vivo (Cong et al., 1997). By enhancing both CD80 and CD86, without affecting MHC class II expression, CT may satisfy the initial requirements necessary for T cell activation. The specific inhibition of the Th1-associated costimulatory molecule CD40 by CT may play a role in enhancement of a Th2/Tr1 response in vivo by removing an important signal for IL-12p70 production by DCs. Not only is CD40 associated with the direct promotion of Th1 cell differentiation through IL-12p70 induction (Cella et al., 1996), lack of CD40 expression on DCs was shown to result in the suppression of a primed immune response via an IL-10-dependent mechanism (Martin et al., 2003). This data may highlight an additional method by which CT induces Tr1 cells, through the reduction of CD40 expression. CT also enhances the expression of the Th2-associated molecule OX40 on DCs (Lavelle et al., 2003). This may play an additional role in the induction of Ag-specific Th2 cells in vivo; however, studies by de Jong and colleagues demonstrate that OX40-ligand, also induced by CT on DCs, was not necessary for Th2 cell polarization by CT-treated DCs (de Jong et al., 2002). Taken together, these
results indicate the ability of CT to induce the maturation of DCs, with a phenotype distinct from that induced by Th1-promoting factors, such as LPS, illustrating a possible mechanism whereby CT can promote the differentiation of naïve T cells into effector Th2/Tr1 cells *in vivo*.

In addition to the direct influence of DCs on T cell differentiation, the cytokine milieu at the site of DC/T cell interaction also plays a fundamental role in the outcome of the T cell response (reviewed in Abbas et al., 1996; Constant and Bottomly, 1997). IL-12, produced by macrophages and DCs, was thought to be the principal cytokine involved in the differentiation of naïve CD4^+ T cells to Th1 cells (Trinchieri, 1995). However, evidence is emerging to suggest roles for additional cytokines, such as IL-23, IL-27, IL-18 and IFN-γ, in the induction and regulation of Th1 cell development (Szabo et al., 2003). On the other hand, IL-4, along with IL-10 and IL-6, are involved in the induction of Th2 cell differentiation (Abbas et al., 1996; Iwasaki and Kelsall, 1999; Rincon et al., 1997). In addition, IL-10 produced by the APC in the absence of IL-12, has been associated with the induction of Tr1 cells (McGuirk et al., 2002). Exposure of DCs to CT, which is devoid of contaminating endotoxin, did not induce the production of any of the above cytokines *in vitro* (Fig. 3.6). Indeed, the only cytokine or chemokine stimulated by CT was the CXC chemokine MIP-2 (Fig. 3.5). It has been reported that the induction of MIP-2 in response to hydrogen peroxide involves a number of key intracellular signalling molecules, including cAMP and ERK MAP kinase (Jaramillo and Olivier, 2002). CT is a potent activator of cAMP (Spangler, 1992) and therefore suggests a possible mechanism for the induction of MIP-2. It is also interesting to note that MIP-2 induced by CT is not detected until about 12 hours of culture (E.C. Lavelle, unpublished observations), whereas LPS-induced MIP-2 can be detected as early as
30 min in the supernatant of stimulated macrophages (Kopydlowski et al., 1999) and DCs (E.C. Lavelle, unpublished observations), which may point towards the activation of additional signalling pathways by CT. MIP-2 is a potent inducer of neutrophil activation and their directional migration (Feng et al., 1995) and our group has found that recombinant MIP-2 can enhance immune responses to coadministered Ag (E.C. Lavelle et al., unpublished data). Therefore the induction of MIP-2 by CT may play a role in the adjuvant action of the toxin *in vivo*.

In the presence of TLR-ligands, CT dramatically enhances the production of IL-10 and IL-1β (Fig. 3.6). We have also shown the specific enhancement of IL-6 production by CT (Lavelle et al., 2003). The upregulation of these cytokines by CT has previous been reported for macrophages (Cong et al., 2001). As discussed above, IL-10 and IL-6 expression by DCs influences the differentiation of naïve Th cells into Th2 cells and Tr1 cells. IL-6 can initiate the development of Th2 cells through a number of different mechanisms including 1) the direct induction of endogenous IL-4 (Heijink et al., 2002; Rincon et al., 1997) 2) inhibition of Th1 cell development through direct suppression of IL-12 (Dodge et al., 2003; Takenaka et al., 1997) and 3) up-regulation of SOCS1 (which interferes with STAT1 phosphorylation) expression in activated CD4⁺ T cells (Diehl et al., 2000). Although, IL-1β has classically been defined as a pro-inflammatory cytokine, recent studies have suggested a role for this cytokine in the activation of Th2 cells (Huber et al., 1996) and the development of the Th2-mediated airway hypersensitivity response (Nakae et al., 2003).

In addition to the enhancement of anti-inflammatory and known Th2/Tr1-inducing cytokines, CT inhibits the production of the pro-inflammatory cytokines IL-12p70, IL-12p40, and TNF-α in response to the TLR4-ligand LPS or the TLR9-ligand CpG (Fig. 3.6) as well as the TLR3-ligand poly(I:C) (Lavelle et al., 2003). These
findings are in agreement with a number of different reports (Braun et al., 1999; Cong et al., 2001; Gagliardi et al., 2000) and are consistent with studies of the closely related AB bacterial toxin, heat-labile enterotoxin (LT) from *E. coli*, which also inhibits IL-12p70 (Ryan et al., 2000). Both of these toxins are effective inducers of cAMP and their ability to inhibit inflammatory cytokines has been linked to the elevation of cAMP (Bagley et al., 2002). Indeed, forskolin, a direct activator of adenylate cyclase which induces intracellular increases in cAMP, mimics the effects of CT and LT by abrogating LPS-induced IL-12p70 and TNF-α secretion from human DCs (Gagliardi et al., 2000). Other factors that induce cAMP, such as β2-agonists (Panina-Bordignon et al., 1997), adenosine and A2a receptor agonists ((Panther et al., 2001) or PGE2 (Kalinski et al., 1997) also inhibit IL-12 secretion by human DC, indicating a key role for cAMP in the inhibition of inflammatory cytokines. However, the mechanism of suppression of IL-12 by CT may not be as clear-cut as implied above, since studies carried out by Burkart and colleagues have shown that high doses of the B subunit of CT, which cannot elevate cAMP levels, inhibit LPS-induced TNF-α and IL-12p70 from human PBMC and a monocyte cell line (Burkart et al., 2002), indicating the possibility of an alternative mechanism of suppression employed by CT, which involves the binding of the toxin to its surface receptor.

A direct comparison of the influence of CT and PT on LPS-induced IL-12p70 and IL-10 production from DCs revealed opposing effects, with PT synergising with LPS to enhance IL-12p70 and reduce IL-10, while CT enhances IL-10 and inhibited IL-12p70 (Fig. 3.9). The effect of PT on IL-12 production from DCs observed in this study is consistent with previous findings (Ausiello et al., 2002; de Jong et al., 2002). The opposing effects of these two toxins on cytokine production from DCs *in vitro* correlates with the distinct Ag-specific T cell cytokine profiles observed *in vivo* (Fig.
with CT enhancing Th2/Tr1 and PT enhancing Th1/Th2 responses. Interestingly, a recent study demonstrated that PT treated DCs promotes naïve T cell development towards the Th1-associated phenotype in an IL-12-dependent manner, whereas CT instructed DCs to induce Th2 cells (de Jong et al., 2002).

This study, along with a number of previous reports clearly demonstrates that CT can enhance TLR-ligand-induced IL-10 production while completely suppressing IL-12 from DCs. However, the modulatory effect of CT on LPS-induced responses in vivo has not been reported. Data presented here demonstrates that LPS as an adjuvant significantly enhanced Ag-specific IL-10 and IFN-γ, but did not significantly enhance the Th2-associated cytokines, IL-4, IL-5 and IL-13. This Th1-biased response with high IL-10 enhanced by LPS was also reflected in the serum antibody response, with a high IgG2a:IgG1 ratio. This result is consistent with a previous report which showed a Th1 response with high IL-10 in response to E. coli LPS in vivo (Pulendran et al., 2001). It is important to note that the LPS used in this study is E. coli LPS, which signals through TLR4 (Ozinsky et al., 2000) and induces high IL-12 production from DCs in vitro (Fig. 3.6), as LPS from P. gingivalis, which signals through TLR2 (Hirschfeld et al., 2001) and does not induce IL-12 from CD8α+ DCs (Pulendran et al., 2001), induces an immune response characterised by IL-5, IL-10 and IL-13 but low IFN-γ in vivo (Pulendran et al., 2001). The ability of E. coli LPS to induce IL-12 production from macrophages and DCs in vitro and bias the in vivo response towards a Th1 phenotype is consistent with reports demonstrating an important role for IL-12 in LPS-induced IFN-γ (Macatonia et al., 1995; Magram et al., 1996).

LPS also enhances T cell derived IL-10 in vivo, although not as efficiently as CT (Fig. 3.10). This observation is consistent with a previous study published by our own group illustrating that TLR4 signalling is implicated in the induction of IL-10 in
response to *Bordetella pertussis* (Higgins, et al., 2003). It is not clear why bacterial products should enhance both pro- and anti-inflammatory cytokines *in vivo* but may represent a mechanism employed by the host to limit the severity of an inflammatory response induced by bacterial LPS. Indeed, the severity of disease and inflammation is exacerbated in IL-10-defective mice infected with *E. coli* (Sewnath et al., 2001). However, IL-10 induction is not entirely dependent on TLR4 signalling, as CT (Fig. 3.10) and *P. gingivalis* LPS (Pulendran et al., 2001) which does not signal through TLR4, can induce Ag-specific IL-10 production *in vivo.*

The potential mechanisms involved in the enhancement of IL-10 by CT on its own *in vivo* are not clear, as this toxin requires the presence of an additional signal *in vitro* to enhance the production of this cytokine from DCs. However, it is possible that additional signals may be provided *in vivo* in the form of endogenous TLR-ligands, such as heat shock proteins (hsp). These molecules are natural chaperones but are released by necrotic cells, and under conditions of stress their expression is upregulated. Recently, a study carried out on human hsp60 suggests that this molecule induces macrophage activation via a TLR4-dependent mechanism (Ohashi et al., 2000).

The data presented in this chapter also suggests that CT can switch the response induced by LPS *in vivo* from a Th1-type response to a more mixed Th1/Th2 response, with a significant increase in Ag-specific IL-4, IL-13 and IL-10 and a significant decrease in IFN-γ production compared to mice immunized with KLH and LPS only. This is also reflected in the antibody responses, with an increase in Ag-specific IgG1 in mice immunized with KLH and LPS and CT compared to the mice that received KLH and LPS. This switch *in vivo* reflects the modulation of LPS-induced cytokine production from DCs by CT observed *in vitro,* which includes an
increase in IL-1β, IL-6 and IL-10, and an inhibition of IL-12p70, IL-12p40 and TNF-α. The partial reduction of IFN-γ may be due to the suppression of IL-12 production by CT from DCs; however other Th1-promoting cytokines, for example IL-23 or IL-27 may not be affected by CT and therefore may explain why complete inhibition of IFN-γ was not observed. Indeed, addition of LPS in vivo significantly enhanced KLH-specific IFN-γ production by T cells from mice immunized with KLH and CT, suggesting that CT does not suppress all factors that promote Th1-cell development in vivo. These results suggest that IL-12 plays only a partial role in the enhancement of T cell specific IFN-γ production by LPS in vivo and that other Th1-promoting factors are involved. This may also explain the enhancement of Ag-specific IFN-γ by CT reported in some studies, which may involve additional signals by endogenous TLR ligands in vivo.

The inhibition of KLH-specific Th2 cytokines in mice immunized with KLH and CT and LPS, probably reflects the increased levels of IFN-γ-producing T cells induced with this combination, compared with mice that received KLH and CT only. IFN-γ exerts an inhibitory influence on the differentiation and effector function of Th2 cells (Mosmann and Coffman, 1989). However, an assessment of the Ag-specific cytokine production induced in IFN-γ-deficient mice or in the presence of anti-IFN-γ antibodies by CT and LPS would be required to confirm this suggestion. It is interesting to note that compared with mice immunized with KLH and LPS as adjuvant, the presence of CT and LPS in vivo significantly enhances Ag-specific IL-10, IL-13 and to a lesser degree IL-4 and IL-5. This illustrated the potent Th2-inducing capacity of CT, even in the presence of high levels of IFN-γ.

In conclusion, the data presented in this chapter confirms that CT is a Th2/Tr1 promoting agent in vivo and can modulate DCs to adopt a DC2/DCr phenotype in
**vitro**. In addition, CT can partially suppress a Th1-type immune response induced *in vivo*. The next step was to examine the role of the MAP kinase signalling family in the modulation of DCs *in vitro*.
Chapter four

Effect of CT on the MAP kinase signalling family in DCs
Chapter 4

4.1 Introduction

In the previous chapter, the ability of CT to activate DCs and selectively enhance the production of certain chemokines and cytokines while suppressing others was described. However, the signalling pathways involved in the modulation of DCs by CT are not yet known. Since the discovery of the TLR family, many of the signalling pathways involved in the induction of DC maturation and cytokine production in response to a variety of microbial products, such as LPS, have been described (Akira et al., 2001). Two of the main pathways involved in the induction of cytokine transcription in DCs are the NFκB pathway and the MAP kinase signalling pathway.

This study focused on the MAP kinase pathway, one of the most ancient signal transduction pathways in mammalian cells (Chang and Karin, 2001; Dong et al., 2002). The MAP kinase signalling pathway consists of a number of serine/threonine kinases that link signal transduction events from the cell surface to the nucleus via the phosphorylation of transcription factors (Karin, 1992; Robinson and Cobb, 1997). Three distinct MAP kinase signalling cascades have been identified to date; the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase pathways (Chang and Karin, 2001). A number of reports have described a role for MAP kinase activation in the maturation of DCs (Aicher et al., 1999; Sato et al., 1999), and a critical role for the MAP kinases in regulation of the Th1/Th2 balance in T cells has recently been described (Rincon, 2001). Emerging evidence also suggests a role for these signalling molecules in regulating cytokine production from macrophages and/or DCs (Hacker et al., 1998; Yi et al., 2002).
However, a comprehensive understanding of the action of these kinases in immune function remains unclear.

The NFκB signalling pathway has been shown to play an important role in the induction of proinflammatory cytokines, including IL-12; however, members of the MAP kinase signalling family have been associated with the induction of IL-10 (Yi et al., 2002). Therefore, because CT is a potent enhancer of IL-10, the role of the MAP kinase signalling family in the effects of CT on DCs was assessed in this chapter. The ability of CT to activate ERK and p38, compared with LPS, was assessed. The results show that CT can activate ERK but not p38 in DCs, with very different kinetics when compared with LPS. Specific inhibitors of ERK and p38 were used to investigate the involvement of these two kinases in the induction of DC maturation and modulation of LPS-induced cytokine production by CT. The results demonstrate a role for ERK in the induction of MIP-2 and enhancement of CD80 expression by CT and also in the modulation of LPS-induced IL-10 and IL-12 production. The data also shows that CT induces the expression of the immediate early gene product c-fos, which has recently been implicated in the modulation of IL-10 and IL-12 (Dillon et al., 2004). Therefore, the data presented here demonstrates that CT directly activates a member of the MAP kinase family in DCs and provides functional relevance for this induction in the modulation of the immune response by this toxin.
4.2 Results

4.2.1 CT induces ERK phosphorylation in DCs

To investigate whether the ERK MAP kinase pathway is involved in CT induced signal transduction in DCs, ERK activation was assessed by measuring its phosphorylation by western blotting using a phospho-specific ERK 1/2 antibody. As seen in a representative immunoblot in the upper panel of Fig. 4.1, CT enhanced ERK phosphorylation in DCs. The lower panel shows the same samples stripped and probed with an antibody specific for total ERK1, demonstrating equal amounts of ERK protein in each sample.

To determine the optimal dose of CT required to induce ERK phosphorylation, DCs were exposed to increasing doses of CT. 10 ng/ml of CT enhanced the phosphorylation levels of ERK 1/2 compared with untreated cells after 30 min of stimulation (Fig. 4.2, lane 2 vs lane 1), though 100 ng/ml of CT was the optimal dose for ERK phosphorylation at this time point (Fig. 4.2; lane 4). However, when compared with the levels of phosphorylated ERK induced by LPS at this time (Fig. 4.2; lane 7), CT, at any of the doses examined, was not as potent as LPS. The lower panel indicates the amount of ERK1 in each sample and is used to verify equal loading of sample.

An examination of the kinetics of ERK phosphorylation revealed an interesting difference in the magnitude and duration of phosphorylation of this kinase by CT when compared to LPS. CT induced the phosphorylation of ERK 30 min after stimulation (Fig. 4.3A, lane 2), an effect that was sustained at a relatively high level for at least 8 hours after addition of the toxin (Fig. 4.3A, lanes 2 – 6). In contrast, LPS strongly stimulated a rapid increase in the levels of ERK phosphorylation (Fig. 4.3B, lane 2) but this increase was transient, with the levels of phosphorylated ERK
Figure 4.1  CT induces ERK phosphorylation in DCs
Immature DCs were stimulated with or without CT (100 ng/ml) for 2 hours. Medium was removed and 100 µl of 1x sample buffer was added to the wells. The cells were harvested, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phosphorylated (phospho)-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel shows a representative immunoblot of phosphorylated ERK, while the lower panel shows its total ERK1, indicating equal loading of samples.
Figure 4.2  CT induces phosphorylation of ERK in DCs at concentrations as low as 10 ng/ml

Immature DCs were stimulated with 10-1000 ng/ml of CT (lanes 1 to 6) as indicated above or with 100 ng/ml of LPS (lane 7) as a positive control for 30 mins. Cells were lysed in 100 μl of 1x sample buffer in the wells, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel shows phosphorylated ERK, while the lower panel shows total ERK1, indicating equal loading of samples. Identical results were obtained in two further experiments.
declining towards basal levels within 60 min of stimulation (Fig. 4.3B, lane 3). The lower panels in both A and B show the same samples after they were stripped and reprobed for total ERK1, indicating equal protein loading. Therefore, CT induces a sustained ERK signal in DCs, while LPS-induced ERK activation is potent but transient.

4.2.2 Role for the enzymatic activity of CT in the induction of phosphorylated ERK

CT is a potent elevator of intracellular concentrations of cAMP via its enzymatic activity (Wacholtz et al., 1991). However, the ability of cAMP to activate or inhibit ERK remains controversial, with evidence in the literature supporting both possibilities, depending on the cell type in question (Busca et al., 2000; Schmidt et al., 1999). In addition, a link between cAMP and ERK has not been fully explored in DCs (Galgani et al., 2004).

The role of the enzymatic activity of CT in the induction of phosphorylated ERK 1/2 in DCs was assessed by utilising the recombinant B subunit of CT (CTB). As previously demonstrated, incubation of DCs with CT for 30 mins induced the phosphorylation of ERK (Fig. 4.4, lane 2). In contrast, incubation of DCs with CTB, which does not enhance cAMP, did not enhance the levels of phosphorylated ERK above those detected in unstimulated DCs at this time point (Fig. 4.4, lane 3). Equal levels of total ERK1 shown in the lower panel indicate that equal levels of protein were in each sample. This result suggests a role for cAMP in the activation of ERK by CT in DCs.
Figure 4.3  CT induces prolonged phosphorylation of ERK in DCs

Immature DCs were stimulated with 100 ng/ml of CT (A) or 100 ng/ml of LPS (B) for 0, 0.5, 1, 2, 4, 8 or 12 hours (lanes 1 to 7). Cells were lysed in 100 μl of 1x sample buffer in the wells, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel of A or B shows phosphorylated ERK, while the lower panel of A or B shows total ERK1, indicating equal loading of samples. Identical results were obtained in three further experiments.
Figure 4.4  CTB fails to induce phosphorylation of ERK

Immature DCs were treated with medium (lane 1), 100 ng/ml of CT (lane 2) or 1 μg/ml of CTB (lane 3) for 30 mins. Cells were lysed in 100 μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel shows phosphorylated ERK, while the lower panel shows total ERK1, indicating equal loading of samples. Identical results were obtained in two further experiments.
4.2.3 CT does not induce p38 expression in DCs

The induction of p38 by CT in DCs was assessed by measuring the phosphorylation of this MAP kinase by western blotting using a phospho-specific p38 antibody. As a comparison, the levels of phosphorylated p38 induced by LPS were also assessed. Resting DCs expressed very low levels of phosphorylated p38 (Fig. 4.5; lane 1), whereas LPS effectively induced p38 phosphorylation (Fig. 4.5B) with high levels of phosphorylated p38 detected 30 min after stimulation (Fig. 4.5B, lane 2). This activation of p38 by LPS persisted for about 4 hours, albeit at much lower levels that those detected at 30 min (Fig. 4.5B, lanes 2 to 5) and levels of phosphorylated p38 returned to basal levels 6 hours after LPS stimulation (Fig. 4.5B, lane 6). In contrast, CT did not induce the phosphorylation of p38 in DCs at any of the time points examined (Fig. 4.5A, lanes 2 to 6). Phosphorylation of p38 was detected in the same DC preparation stimulated with LPS (Fig. 4.5A, lane 7) and equal levels of total p38 were detected in these samples (Fig. 4.5A; lower panel). Therefore, LPS induces rapid and potent phosphorylation of p38, whereas CT does not activate this kinase in DCs.

The possibility that CT may modulate the expression of p38 induced by LPS was assessed by incubating DCs with CT for 2 hours prior to the addition of a range of doses of LPS (1-100 ng/ml) for 20 mins. CT did not activate p38 (Fig. 4.6; lane 2) while increasing doses of LPS resulted in the increased activation of p38 (Fig. 4.6; lane 3, 5 and 7). Incubation of DCs with CT prior to LPS stimulation did not alter the phosphorylation levels of p38 compared with that observed with LPS alone at 1 ng/ml or 10 ng/ml (Fig. 4.6; compare lanes 4 and 3, lanes 6 and 5). Pre-incubation of DCs with CT for 2 hours prior to stimulation with 100 ng/ml of LPS slightly increased the
Figure 4.5  LPS but not CT induce phosphorylation of p38 in DCs

Immature DCs were treated with 100 ng/ml of CT (A - top panel), or 100 ng/ml of LPS (B - middle panel) for 0, 0.5, 1, 2, 4 or 8 hours (lane 1 - 6). Cells treated with 100 ng/ml of LPS for 30 mins were used as a positive control (A; lane 7). Cells were lysed in 100 μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-p38. The nitrocellulose was then stripped and assayed for total p38. The upper and middle panels show phosphorylated p38, while the lower panel shows total p38, indicating equal loading of samples. Identical results were obtained in three further experiments.
Figure 4.6  CT does not inhibit LPS-induced phosphorylation of p38

Immature DCs were treated with medium (lanes 1, 3, 5 and 7) or 100 ng/ml of CT (lanes 2, 4, 6 and 8) for 2 hours followed by 1 ng/ml of LPS (lanes 3 and 4), 10 ng/ml of LPS (lanes 5 and 6) or 100 ng/ml (lanes 7 and 8) for 20 mins. Cells were lysed in 100μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-p38. The nitrocellulose was then stripped and assayed for total p38. The upper and middle panels show phosphorylated p38, while the lower panel shows total p38, indicating equal loading of samples. Identical results were obtained in two experiments.
phosphorylation of p38 compared to LPS alone (Fig. 4.6; compare lanes 8 and 7). However, further analysis is required to verify this observation.

**4.2.4 Effect of selective inhibitors of the ERK signalling cascade on CT or LPS stimulated MAP kinase activity**

Two selective and potent inhibitors of the ERK MAP kinase signalling cascade were used in this study to determine the functional activities mediated by the activation of ERK MAP kinase. U0126 is a pharmacological inhibitor that mediates its effect on ERK by inhibiting the activated, phosphorylated form of its upstream activator MEK 1/2 (Favata et al., 1998). The other inhibitor employed in this study was PD98059, which primarily inhibits MEK 1/2 activation by blocking access to its activating enzyme (Dudley et al., 1995).

Fig. 4.7 shows that U0126, when added to DCs for 1 hour prior to stimulation with CT or LPS, inhibits both CT- and LPS-induced phosphorylated ERK. 2.5 μM of U0126 was sufficient to completely inhibit the induction of phosphorylated ERK by CT (Fig. 4.7; compare lanes 4 and 5), as well as LPS (Fig. 4.7; compare lanes 7 and 8). U0126 also reduced the basal levels of activated ERK in these cells (Fig. 4.7; compare lane 1 with 2 and 3). The lower panel shows total ERK1 levels in these samples indicating equal loading of protein.

A similar trend was observed with PD98059. Pre-incubation with 25 or 50 μM of PD98059 completely inhibited CT-induced phosphorylated ERK in DCs (Fig. 4.8, compare lane 4 with 5 and 6). The lower panel indicates that this difference is not due to unequal loading of protein as the same amount of ERK1 is present in each sample.
Figure 4.7  Phosphorylation of ERK by CT or LPS is inhibited by the MEK 1/2 inhibitor U0126

Immature DCs were treated with medium (lanes 1, 4 and 7), 2.5 μM of U0126 (lanes 2, 5 and 8) or 5 μM of U0126 (lanes 3, 6 and 9) for 1 hour prior to the addition of medium (lanes 1, 2, 3), CT (100 ng/ml; lanes 4, 5 and 6) or LPS (100 ng/ml; lanes 7, 8 and 9) for 2 hours. Cells were lysed in 100 μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel shows phosphorylated ERK, while the lower panel shows total ERK1, indicating equal loading of samples. Results are representative of two experiments.
Figure 4.8  The MEK 1/2 inhibitor PD98059 inhibits CT induced phospho-ERK

Immature DCs were treated with medium (lanes 1 and 4), or with the MEK 1/2 inhibitor PD98059 (25 μM, lanes 2 and 5 or 50 μM, lanes 3 and 6) for 1 hour prior to the addition of medium (lanes 1, 2, 3) or CT (100 ng/ml; lanes 4, 5 and 6) for 2 hours. Cells were lysed in 100 μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs specific for phospho-ERK. The nitrocellulose was then stripped and assayed for ERK1. The upper panel shows phosphorylated ERK, while the lower panel shows total ERK1, indicating equal loading of samples. Results are representative of two experiments.
4.2.5 CT enhances DC expression of the immediate-early gene product c-fos

Recent studies have suggested that sustained ERK signalling results in the activation and stabilisation of the immediate-early gene product c-fos in a fibroblast cell line (Murphy et al., 2002). This may provide a mechanism by which sustained ERK signals could be translated into a specific biological outcome. Therefore, the ability of CT to induce c-fos expression in DCs was assessed by measuring c-fos levels in whole cell lysates by western blotting using a specific anti-c-fos antibody. In addition, the kinetics of c-fos expression was assessed. c-fos expression was barely detectable in resting DCs (Fig. 4.9; lane 1). However, 1 hour of stimulation with CT greatly enhanced c-fos expression in DCs (Fig. 4.9A; compare lanes 3 and 1). This enhancement of c-fos by CT was further increased up to 4 hours (Fig. 4.9A; lane 5), after which time the levels of c-fos detected in the cells gradually diminished, until basal levels of c-fos expression were reached after 10 hours of stimulation (Fig. 4.9A; lane 8). In contrast, stimulation of DCs over the same period of time with LPS did not result in the consistent enhancement of c-fos expression (Fig. 4.9B). c-fos expression was slightly enhanced after 1 hour and 10 hours of stimulation with LPS (Fig. 4.9B; lanes 3 and 8) but this signal was not sustained. The bottom panel of A and B shows β-actin expression in the same blots after they were stripped, indicating equal loading of the samples. These data unequivocally illustrates the induction of the immediate-early gene product c-fos in DCs in response to CT but not in response to LPS.

It has been reported that c-fos activation was dependent on ERK phosphorylation in a fibroblast cell line (Murphy et al., 2002); therefore, we sought to determine whether the induction of c-fos by CT in this study was due to sustained activation of ERK (Fig. 4.3). As previously described, c-fos expression was enhanced
Figure 4.9  CT but not LPS induces c-fos in DCs

Immature DCs were stimulated with CT (A; 100 ng/ml) or LPS (B; 100 ng/ml) for 0.5, 1, 2, 4, 6, 8 or 10 hours as indicated above (lanes 2 to 8). Cells were lysed in 100 µl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs for c-fos as shown in the upper panel. The nitrocellulose was then stripped and assayed for β-actin (lower panel) indicating equal loading. Identical results were obtained in three further experiments.
in DCs that were stimulated with CT for 2 hours (Fig. 4.10; compare lane 2 with 1). Incubation with low doses of the MEK1/2 inhibitor U0126 for 1 hour prior to stimulation with CT did not alter the induction of c-fos (Fig. 4.10, lane 3 and 4). However, pre-incubation of DCs with U0126 at 5 μM prior to stimulation with CT resulted in the inhibition of CT-induced c-fos expression (Fig. 4.10; compare lane 5 with 2). This concentration of U0126 is the same dose that was used by Murphy et al. (2002) to show that c-fos can function as a sensor for ERK signal duration. The lower panel, indicating β-actin levels in these samples, demonstrates that the differences observed in c-fos levels are not due to different protein concentrations in the samples. Therefore, the induction of c-fos in DCs by CT appears to partially involve the activation of ERK.

4.2.6 The role of ERK and p38 in the up-regulation of CD80 and CD86

To gain insight into the functional relevance of the induction of ERK by CT, we first examined the possible involvement of this kinase in the upregulation of the costimulatory molecules CD80 and CD86 on DCs, with the use of the highly selective synthetic inhibitor of ERK, U0126. Immature DCs were incubated for 1 hour with increasing doses of U0126 (1.25 – 5 μM) prior to stimulation with CT (1 μg/ml). The expression of CD80 and CD86 was determined on the surface of these cells by flow cytometry 24 hours later (Fig. 4.11). As previously described, stimulation of DCs with CT enhanced the expression of CD80 and CD86. Incubation of DCs with increasing doses of U0126 alone did not modulate the expression of CD80. However, CD86 expression on unstimulated DCs was slightly upregulated in the presence of U0126 (5 μM), with the MFI increasing from 4.03 (untreated cells) to 6.44. In contrast, the upregulation of CD80 expression by CT was reduced with increasing
Figure 4.10 The MEK1/2 inhibitor U0126 suppresses CT-induced c-fos expression in DCs

Immature DCs were incubated with 1.25 µM (lane 3), 2.5 µM (lane 4) or 5 µM (lane 5) of U0126 1 hour prior to stimulation with 100 ng/ml of CT (lane 2-5) for 2h. Cells were lysed in 100 µl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose and probed with Abs for c-fos as shown in the upper panel. The nitrocellulose was then stripped and assayed for β-actin (lower panel) indicating equal loading.
Figure 4.11 Inhibition of ERK reduces CD80 and enhances CD86 upregulation by 
CT in DCs

Immature DCs were stimulated with medium or the ERK inhibitor U0126 (1.25 - 5 μM) 
for 1 hour prior to stimulation with CT (1 μg/ml) or medium only. After 24 h incubation, 
cells were washed and stained with antibodies specific for CD80, CD86 or with isotype-
matched control antibodies. Immunofluorescence is shown for DCs treated in the 
presence (solid black line) or absence (gray histograms) of U0126 compared to untreated 
DCs (dotted black line). The isotype control is shown in the control histogram (grey 
line). The numbers on the right of each histogram refer to the MFI of the treated cells in 
the presence of the inhibitor.
doses of the ERK inhibitor. The MFI of cells incubated with CT alone was 321.97, which was reduced in a dose dependent manner to 216.74 (5 μM) with increasing doses of U0126 (1.25 - 5 μM). In contrast, the upregulation of CD86 by CT was further enhanced in the presence of the ERK inhibitor. This enhancement was observed with the lowest dose of the inhibitor examined (1.25 μM) and maintained with increasing doses of the inhibitor. Therefore, inhibition of ERK results in the reduction of CT-induced CD80 expression but enhancement of both basal and CT-induced CD86 expression on DCs.

The role of p38 was also examined by pre-incubating DCs with the p38 inhibitor SB203580 (0.1 – 10 μM) for 1 hour prior to stimulation with CT (Fig. 4.12). The expression of CD80 on unstimulated DCs was modestly upregulated with increasing doses of the p38 inhibitor. Inhibition of p38 with SB203580 had a marginal suppressive effect on the enhancement of CD80 by CT, with the MFI decreasing from 321.97 to 276.32 in the presence of 10 μM of the inhibitor. Inhibition of p38 has a minor enhancing effect on the expression of CD86 on the surface of unstimulated DCs. Upregulation of CD86 by CT was also enhanced in the presence of SB203580. The MFI of CT-treated DCs increased from 7.64 to 9.22 with pre-incubation with 0.1 μM of the inhibitor, and this enhancement was maintained with increasing doses of the inhibitor. Therefore, inhibition of p38 results in a marginal decrease in CT-enhanced CD80 expression and a slight increase in CD86 expression.

4.2.7 The role of ERK and p38 in the modulation of LPS-induced chemokine and cytokine production by CT
Figure 4.12 SB203580 marginally enhances CD86 expression in DCs
Immature DCs were stimulated with medium or the p38 inhibitor SB203580 (0.1 – 10 μM) for 1 hour prior to stimulation with CT (1 μg/ml) or medium only. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86 or with isotype-matched control antibodies. Immunofluorescence is shown for DCs treated in the presence (solid black line) or absence (gray histograms) of U0126 compared to untreated DCs (dotted black line). The isotype control is shown in the control histogram (grey line). The numbers on the right of each histogram refer to the MFI of the treated cells in the presence of the inhibitor.
Previous reports have suggested a role for the ERK signalling pathway in the induction of chemokines in response to inflammatory stimuli (Marumo et al., 1999) and having demonstrated that CT is an effective inducer of MIP-2 in DCs, the role of ERK 1/2 in the induction of MIP-2 by CT was examined. DCs were incubated for 1 hour with increasing doses of the specific ERK inhibitors, U0126 or PD98059, before stimulation with CT (or LPS as a control). Doses as low as 0.8 \( \mu \text{M} \) of U0126 significantly reduced the induction of MIP-2 by CT, with complete suppression of the production of this chemokine in response to CT at a dose of 6.5 \( \mu \text{M} \) (Fig. 4.13A). Interestingly, U0126, even at the highest dose examined, did not significantly affect the levels of MIP-2 induced by LPS. Consistent with this observation, cells incubated with PD98059 (12.5 \( \mu \text{M} \) – 50 \( \mu \text{M} \)) also significantly reduced MIP-2 production in response to CT (Fig. 4.13B). Again, this inhibitor did not affect LPS-induced MIP-2 expression from DCs. In contrast, inhibition of p38, with the inhibitor SB203580, did not significantly modulate the induction of MIP-2 by CT or LPS (Fig. 4.14). Altogether, this data demonstrates an important and specific role for the ERK pathway, but not p38, in CT-induced MIP-2 production in DCs.

To further analyse the role of ERK 1/2 in the immunomodulatory activity of CT, DCs were incubated with increasing doses of U0126 followed by stimulation with CT in the presence or absence of LPS (Fig. 4.15). CT did not induce IL-10, TNF-\( \alpha \), IL-12p70 or IL-12p40 production when incubated alone or in the presence of U0126. Incubation of DCs with 1.25 \( \mu \text{M} \) of U0126 resulted in the significant inhibition of LPS-induced IL-10 production (Fig. 4.15). This inhibition was seen over a range of doses of the inhibitor. In addition, the synergistic activity of CT and LPS on IL-10 production was also reduced, with similar levels of IL-10 detected in DCs stimulated with CT and LPS in the presence of U0126 as that seen with LPS alone.
Figure 4.13 The pERK inhibitors (A) U0126 and (B) PD98059 inhibit CT-induced but not LPS-induced MIP-2 expression in DCs

Immature DCs were pre-incubated with U0126 (0.8μM – 13μM) or PD98059 (3.1 – 50 μM) for 1h prior to addition of medium, CT (1 μg/ml) or LPS (10 ng/ml) for 15 hours. MIP-2 concentrations were determined in supernatants by ELISA. Results are means (± SD) of triplicate assays and are representative of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (CT vs CT plus inhibitor).
Figure 4.14 The p38 inhibitor SB203580 has no effect on CT- or LPS-induced MIP-2 expression in DCs

Immature DCs from BALB/c mice were pre-incubated with medium, 0.1 μM, 1 μM or 10 μM of SB203580 for 1 hour before addition of medium (untreated), CT (1 μg/ml) or LPS (10 ng/ml) for 15 hours. MIP-2 concentrations were determined in supernatants by ELISA. Results are means (± SD) of triplicate assays and are representative of two independent experiments.
Figure 4.15 The effect of the ERK 1/2 inhibitor U0126 on LPS- and CT plus LPS-induced cytokine production

Immature DCs were pre-incubated with the phospho-specific ERK inhibitor U0126 (1.25 μM, 2.5 μM or 5 μM) for 1 hour followed by stimulation with CT (100 ng/ml), LPS (100 ng/ml) or CT and LPS for 6 hours (TNF-α) or 24 hours (IL-10, IL-12p70 and IL-12p40). IL-10, TNF-α, IL-12p70 and IL-12p40 concentrations were determined in supernatants by ELISA. Results are means (± SD) of triplicate assays and are representative of three independent experiments. *, p< 0.05; ***, p < 0.001 (LPS vs LPS + inhibitor); ##, p < 0.01; ###, p < 0.001 (CT + LPS vs CT + LPS + inhibitor).
Pre-incubation with U0126 also inhibited the production of LPS-induced TNF-α (Fig. 4.15). Although co-incubation of LPS with CT suppresses TNF-α production from DCs, this suppression was further enhanced in the presence of the inhibitor (p < 0.001). In contrast to IL-10, the induction of IL-12p70 by LPS was enhanced in the presence of the ERK inhibitor U0126, reaching significance at a dose of 5 μM (Fig. 4.15). CT completely inhibited the production of LPS-induced IL-12p70 and inhibition of ERK did not affect this suppression. In addition, LPS-induced IL-12p40 was also enhanced by U0126, although not to a significant level (Fig. 4.15). The inhibition of LPS-induced IL-12p40 by CT was not affected by the presence of this inhibitor. These data demonstrated that ERK phosphorylation plays an important positive role in the induction of IL-10 and has an inhibitory role in IL-12 production in response to LPS or CT plus LPS.

The role of p38 in IL-10, TNF-α, IL-12p70 and IL-12p40 production induced by CT plus LPS was also examined. LPS-induced IL-10 and TNF-α production was greatly suppressed by inhibition of p38 with SB203580 (Fig. 4.16). Doses as low as 0.1 μM of the inhibitor significantly suppressed the production of both of these cytokines in vitro. Although, CT suppressed TNF-α production by LPS, the presence of SB203580 further reduced the levels of TNF-α secreted by the DCs. In addition, the p38 inhibitor SB203580 also significantly inhibited IL-10 production from DCs in response to CT and LPS.

In contrast, incubation of DC with SB203580, prior to stimulation with LPS, resulted in significant enhancement of both IL-12p40 and IL-12p70 compared with cells that were stimulated with LPS alone (Fig. 4.16). The complete inhibition of LPS-induced IL-12p70 by CT was not affected by the presence of SB203580; however, inhibition of p38 resulted in the partial reversal of the inhibitory effects of
Figure 4.16 The p38 inhibitor SB203580 inhibits LPS- or CT plus LPS-induced IL-10 and TNF-α production but enhances LPS-induced IL-12p70 and IL-12p40 in addition to reversing the suppression of LPS-induced IL-12p40 by CT.

Immature DCs were pre-incubated with 0.1 μM, 1 μM or 10 μM of the phospho-specific p38 inhibitor SB203580 for 1 hour followed by stimulation with CT (100 ng/ml), LPS (100 ng/ml) or CT and LPS for 6 hours (TNF-α) or 24 hours (IL-10, IL-12p70 and IL-12p40). IL-10, TNF-α, IL-12p70 and IL-12p40 concentrations were determined in supernatants by ELISA. Results are means (± SD) of triplicate assays and are representative of three independent experiments. **, p < 0.01; *** p < 0.001 (LPS vs LPS + inhibitor); #, p < 0.05, ##, p < 0.01, ###, p < 0.001 (CT + LPS vs CT + LPS + inhibitor).
CT on LPS-induced IL-12p40. Incubation of DCs with SB203580 (1 μM or 10 μM) prior to stimulation with CT plus LPS resulted in the production of similar levels of IL-12p40 compared with cells treated with LPS alone. These results indicate that (1) p38 activation is essential for IL-10 and TNF-α production, (2) the inhibition of TNF-α by CT is independent of p38 and (3) activation of p38 in our system provides an inhibitory signal for IL-12 production in response to LPS.
4.3 Discussion

In an effort to understand the mechanisms involved in the modulation of DC maturation and cytokine production by CT, the role of the MAP kinase signalling cascade was examined. The signalling pathways induced by a variety of microbial stimuli in innate cells, especially DCs, have been extensively characterised (Akira et al., 2001). Signalling via all of the TLRs identified to date culminates in the activation of a number of signalling cascades, including NFκB and the MAP kinase cascade (Kopp and Medzhitov, 1999), indicating the importance of both of these signalling pathways in the activation of the innate immune system. A number of recent reports have linked members of the MAP kinase signalling family with the modulation of key regulatory cytokines; LPS-induced p38 phosphorylation has been associated with IL-12 secretion from monocytes (Lu et al., 1999), while activation of ERK by CpG DNA was shown to induce IL-10 production (Yi et al., 2002). Therefore, these signalling molecules may play a role in the enhancement of IL-10 and the suppression of LPS-induced Th1-promoting cytokine production from DCs by CT.

Consequently, I assessed the role of the ERK and p38 signal pathways in CT-induced cytokine production and modulation of DCs. The results presented in this chapter demonstrate that CT is a potent activator of ERK but not of p38 in DCs. This is the first demonstration of the direct activation of a member of the MAP kinase signalling family by CT in DCs. The complete mechanism involved in the induction of ERK by CT has not yet been defined. However, the data presented here suggests an important role for cAMP in the induction of ERK, as the B subunit of CT, which cannot enhance intracellular levels of cAMP, did not induce phosphorylation of ERK in DCs. A number of recent studies have illustrated an association between cAMP
and ERK. It appears that cAMP can have a positive or negative effect on ERK activation, depending on the cell type studied (Busca et al., 2000; Schmidt et al., 1999). For cAMP to have a positive effect on ERK, it appears that phosphorylation of the Raf isoform B-Raf is required (Stork and Schmitt, 2002). In cells that do not express B-Raf, transfection of B-Raf into these cells converted cAMP from an inhibitor to an activator of ERK (Vossler et al., 1997). Another study demonstrated that activation of ERK in melanocytes by cAMP involved Ras and B-Raf (Busca et al., 2000) providing further evidence for the role of B-Raf in cAMP-induced activation of ERK. Whether DCs express B-Raf and its role in CT-induced ERK activation has still to be defined.

The association between p38 and cAMP has not been examined in much detail. However a recent study demonstrated the induction of p38 in macrophages in response to the soluble cAMP analog dibutyryl cAMP (dBcAMP) (Chio et al., 2004). Furthermore, although 8-Br-cAMP did not enhance LPS-induced p38 levels in DCs as determined by western blotting technique, this cAMP elevating agent was shown to greatly enhance LPS-induced p38 activation by examining the phosphotransferase activity of p38 on an exogenous substrate (Galgani et al., 2004). Therefore, although modulation of LPS-induced p38 activation by CT was not detected in this study using the western blotting technique, it may be possible that enhancement of p38 by CT occurs at low levels and a more sensitive mechanism of detection is required.

LPS strongly induces ERK in a rapid but transient manner (Feng et al., 1999). In contrast, the induction of ERK by CT was greatly prolonged when compared with LPS. Sustained activation of ERK has previously been observed in response to Pam-3-Cys, a synthetic TLR2 agonist, and schistosome egg antigens (SEA), both of which
bias the immune response towards a Th2 pathway (Agrawal et al., 2003). The functional relevance of prolonged ERK activation has previously been described in a number of different cell types. Sustained but not transient activation of ERK has been associated with S phase entry in fibroblasts (Balmanno and Cook, 1999; Weber et al., 1997) while in PC12 cells, sustained ERK activation preceded differentiation into sympathetic-like neurons (Marshall, 1995). Recently a molecular mechanism by which cells can interpret differences in the kinetics of ERK activation has been proposed (Murphy et al., 2002), with the immediate early gene product, c-fos functioning as a sensor for ERK signal duration. c-fos possesses an amino acid sequence, termed the DEF domain, which acts as a docking site for ERK. During prolonged ERK activation, sufficient accumulation of c-fos protein occurs, which is then stabilised by activated ERK, resulting in c-fos-mediated signalling (Murphy et al., 2002). As demonstrated in this study, sustained ERK activation by CT correlated with continued c-fos protein expression in DCs (Fig. 4.3 and 4.9). In comparison, LPS, which induces a strong but transient ERK signal, did not induce c-fos expression. Consistent with this, Pam-3-cys and SEA, but not E. coli LPS can enhance and maintain c-fos expression in DCs (Agrawal et al., 2003). The complete inhibition of CT-induced c-fos expression by 5 μM of the ERK inhibitor U0126, confirmed that c-fos is a downstream effector of ERK (Fig. 4.10). Several cis elements mediate c-fos induction, including a cAMP response element (CRE), which is likely to be occupied by CREB or ATF proteins, indicating a cAMP- and Ca\(^{2+}\)-dependent signalling pathway mediating c-fos induction (Sheng et al., 1991). While Ca\(^{2+}\) signalling induces transient c-fos mRNA, cAMP is a strong inducer of c-fos gene transcription in macrophages (Bravo et al., 1987). However, c-fos requires phosphorylation to prolong its half-life to at least 2 hours, which is carried out by
ERK (Okazaki and Sagata, 1995). Therefore, the prolonged activation of c-fos by CT may be controlled by the induction of this protein by cAMP, which is then stabilized through phosphorylation by sustained ERK activation. Indeed, CT has previously been shown to activate c-fos in astrocytes and fibroblasts in a cAMP-dependent manner (Gabellini et al., 1991).

As discussed in chapter 3, exposure of DCs to CT results in the upregulation of CD80 and CD86 expression on the surface of the cells. Data presented in this chapter shows that the MAP kinase ERK is involved, at least in part, in the upregulation of CD80 expression by CT. Inhibition of ERK resulted in the partial inhibition of CT-induced CD80 expression but had no effect on the basal expression of this costimulatory molecule. In contrast, ERK activation appears to exert an inhibitory role on the expression of CD86, as enhancement of both constitutive and CT-induced CD86 expression was detected in the presence of the ERK inhibitor. Contrary to previous reports, which place CD80 and CD86 expression downstream of the same signalling cascade (Hoebe et al., 2003), these results indicate that these costimulatory molecules have different requirements for ERK in response to CT.

Examination of the human promoter sequence of CD80 identified a binding site for CREB (Fong et al., 1996). The ERK MAP kinase has previously been associated with the complete phosphorylation of CREB in response to nerve growth factor (Xing et al., 1998) or anti-CD3 and anti-CD28 stimulation in T cells (Yu et al., 2001). Therefore, complete phosphorylation of CREB by ERK may contribute to CT-enhanced CD80 expression in DCs. ERK activation may play an inhibitory role in CD86 expression. This may appear to contradict the observations that CT induces ERK and enhances CD86 expression, but CT also inhibits LPS-induced CD86 expression in DCs (Fig. 3.6) (Lavelle et al., 2003). Therefore, in the presence of CT
and LPS, ERK phosphorylation may reach sufficient intensity for the expression of LPS-induced CD86 to be inhibited. In addition, inhibition of ERK resulted in the enhancement of CD86 expression in unstimulated DCs. However, the mechanism involved in the induction of CD86 by CT is still unknown.

p38 does not appear to play a prominent role in the enhancement of CD80 and CD86 expression in response to CT. However, a slight reduction in CT-induced CD80 expression was observed in the presence of the p38 inhibitor. Previous studies have shown that p38 activation is involved in the upregulation of CD80 and CD86 in response to LPS in a number of cell types (Ardeshna et al., 2000; Arrighi et al., 2001; Iijima et al., 2003). However, in contrast to CD80 expression, inhibition of p38 slightly enhanced constitutive and CT-enhanced CD86 expression (Fig. 4.12). Further examination is required to verify the role of p38 in CT-induced CD80 and CD86 expression.

The enhancement of CD80 and CD86 expression on DCs in response to LPS does not appear to involve MyD88 (Kaisho et al., 2002), but an important role for type 1 interferon receptor (IFN-R1) signalling has been demonstrated for CD80/CD86 expression in murine macrophages (Hoebe et al., 2003). This study by Hoebe et al. showed that LPS- and dsRNA-induced upregulation of these costimulatory molecules was dependent on IFN-β and IFN-R1 signalling. However, CT does not induce IFN-α or IFN-β in DCs, and therefore does not activate the IFN-R1 (E.C. Lavelle, unpublished observations), indicating an alternative signal pathway involved in CT-induced upregulation of CD80 and CD86.

In addition to the upregulation of CD80 and CD86 expression, CT induces the production of the CXC chemokine MIP-2 from DCs. Our data clearly shows that blockage of the ERK pathway with either of two inhibitors specific for MERK 1/2,
significantly inhibits CT-induced MIP-2 production, whereas inhibition of p38 had no effect on the production of this chemokine. It is interesting to note, however, that inhibition of ERK signalling in LPS-stimulated DCs did not effect the induction of MIP-2 expression. This indicates that MIP-2 gene expression can be regulated in an ERK-dependent or an ERK-independent manner, depending on the stimulus used. The involvement of ERK in MIP-2 production is in agreement with a previous report demonstrating that MIP-2 gene expression, in response to hydrogen peroxide, was shown to involve the nuclear translocation of the transcription factors NFκB, AP-1 and CREB in an ERK-dependent manner (Jaramillo and Olivier, 2002). Analysis of the transcription factors utilised by CT in the induction of MIP-2 may further contribute to our understanding of the modulation of DCs by this toxin.

Previous studies have shown that activated ERK 1/2 influences the production of IL-10 and IL-12 positively or negatively, respectively, in response to CD40 or CpG (Mathur et al., 2004; Yi et al., 2002). In addition, differential regulation of IL-12 by ERK and p38 has been revealed, with ERK 1/2 activation being implicated in the inhibition of IL-12 whereas p38 phosphorylation promoted the induction of IL-12 (Feng et al., 1999). Our results indicate that inhibition of ERK 1/2 results in the suppression of both IL-10 and TNF-α production, whereas IL-12p70 levels are enhanced in LPS-stimulated DCs. The synergy for IL-10 production between CT and LPS was also reduced in the presence of U0126, indicating a positive role for ERK activation in IL-10 production. This result concurs with a number of recent studies that have also highlighted a role for ERK in the induction of IL-10 through the use of specific inhibitors and ERK1 knockout mice in response to a number of TLR-ligands or CD40 (Dillon et al., 2004; Mathur et al., 2004; Yi et al., 2002). In addition, data presented in this study demonstrates that inhibition of p38 MAP kinase also
suppresses IL-10 production from CT and LPS-stimulated DCs. A role for p38 in the induction of IL-10 has also been shown by a number of other groups (Carneiro-Santos et al., 2002; Foey et al., 1998; Guo et al., 2003). Therefore, both ERK and p38 play a role in IL-10 production from murine DCs. It would appear that p38 is essential for IL-10 production and due to the fact that the CT alone does not induce phosphorylation of this kinase, this may account for the inability of CT to directly induce IL-10. Maximal production of IL-10 appears to require the additional activation of ERK (Yi et al., 2002). Therefore, prolonged ERK activation by CT may be responsible for the enhancement of IL-10 production from LPS-stimulated DCs. Likewise, Pam-3-cys and LPS both induce p38 activation, but Pam-3-cys induces significantly greater levels of ERK in DCs and also induces much higher levels of IL-10, thereby indicating a role for ERK in maximal induction of IL-10 from DCs (Dillon et al., 2004).

This study also illustrates the essential role of both p38 and ERK in the induction of TNF-α production in response to LPS, which is in agreement with a study examining the induction of TNF-α in response to CpG (Yi et al., 2002). The inhibition of TNF-α by CT appears to be independent of both members of the MAP kinase signalling family, as inhibition of ERK or p38 does not restore TNF-α production in the presence of CT plus LPS. In fact incubation of DCs with either SB203580 or U0126 in the presence of CT and LPS resulted in a further inhibition of TNF-α. Further studies are required to understand the mechanism underlying the inhibition of TNF-α by CT. A number of transcription factors are critical for LPS induction of the TNF-α gene, including ATF-2, Ets-1 and -2, Sp1, c-jun, ELK-1 and Egr-1 as well as CREB binding protein (CBP) and p300, indicating a number of
possible pathways by which suppression of TNF-α production may occur (Tsai et al., 2000).

In contrast to the suppression of IL-10 and TNF-α, the ERK 1/2 inhibitor U0126 enhanced LPS-induced IL-12p70 and IL-12p40 production by DCs. This study confirms previous reports indicating that ERK activation suppresses IL-12 production from DCs in response to LPS or CD40 (Feng et al., 1999; Mathur et al., 2004; Puig-Kroger et al., 2001). However, inhibition of ERK did not restore LPS-induced IL-12p70 or IL-12p40 in the presence of CT. Therefore, this would indicate that CT induces an additional ERK-independent suppressive signal on IL-12 production. Similar to the results presented here, IL-12p70 production was not enhanced by abrogating the ERK signal in the presence of Pam-3-cys, but did enhance IL-12p70 in response to LPS (Dillon et al., 2004). Studies carried out by Dillon et al. (2004) also showed that inhibition of c-fos (through the use of c-fos knockout mice) resulted in the enhancement of IL-12p70 and reduction of IL-10 in response to Pam-3-cys, indicating a fundamental role for c-fos in the regulation of IL-12 and IL-10 production. Therefore, the induction of c-fos by CT in DCs may provide one mechanism by which CT can suppress IL-12p70 production and enhance IL-10. However, removal of c-fos in our system would be required to confirm this.

Surprisingly, inhibition of p38 in DCs with its inhibitor SB203580 resulted in the enhancement of both IL-12p70 and IL-12p40 in response to LPS. This finding contradicts a number of previous reports which show that p38 is essential for IL-12 production (Agrawal et al., 2003; Feng et al., 1999; Goodridge et al., 2003; Mathur et al., 2004; Yi et al., 2002). SB203580 appears to be a highly specific inhibitor of p38 activity, illustrated by its failure to affect the activities of a number of other protein kinases (Cuenda et al., 1995). In addition, the dose of the inhibitor required to
enhance IL-12 production was low (between 0.1 μM and 1 μM), strongly suggesting that the effects seen reflect the direct inhibition of p38 activity. Analysis of the murine IL-12p35 promoter by Kollet and colleagues showed a major negative regulatory region situated in the middle of the promoter, which contained AP-1, C/EBP and CREB recognition sequences (Kollet et al., 2001). Therefore, it is possible that p38, through MAPKAPK-2, which phosphorylates and activates CREB (Xing et al., 1998), may have a role in the negative regulation of IL-12p70 production through its action on the promoter for the IL-12p35 subunit. In addition, it is also possible that the enhancement of LPS-induced IL-12 in the presence of SB203680 is due to the inhibition of IL-10 (Fig. 4.15), as inhibition of IL-10 using an anti-IL-10 antibody resulted in the significant enhancement of LPS-induced IL-12p70 production (Lavelle et al., 2003). The inability of the p38 inhibitor to reverse the suppression of IL-12p70 by CT is probably due to the presence of the additional inhibitory signal through c-fos. The role of c-fos as a negative regulator of IL-12 has previously been discussed and is confirmed with a study which utilized macrophages from c-fos knockout mice revealing that loss of c-fos increased LPS-induced IL-12 production (Roy et al., 1999).

In addition to the enhancement of LPS-induced IL-12 production, inhibition of p38 also partially reversed the CT-induced inhibition of IL-12p40. A recent study showed that LPS-induced IL-10 activated STAT3 phosphorylation in DCs, which provided a negative regulatory signal for IL-12p40 gene expression (Hoentjen et al., 2004). Therefore, through inhibition of CT plus LPS-induced IL-10, the p38 inhibitor may partially restore IL-12p40 by removing a regulatory IL-10-induced STAT-3 signal. However, a complete restoration of the IL-12p40 signal was not observed.
indicating the presence of additional CT-induced regulatory signals that are not p38 dependent.

In conclusion, CT induces sustained activation of ERK in DCs, which plays a role in the suppression of IL-12 and enhancement of IL-10 by the toxin. This modulation of cytokine production by CT may also involve the intermediate early gene product c-fos, which is induced and sustained by CT in DCs. Induction of the chemokine, MIP-2, by CT is dependent on ERK activation. Therefore, ERK MAP kinase plays an important role in the modulation of DCs by CT.
Chapter Five

Modulation of T cell responses by CT - the role of the MAP kinase family
Chapter 5

5.1 Introduction

In the previous chapters, it was shown that CT has the ability to act as a potent adjuvant, enhancing a Th2/Tr1 response to coadministered Ag in vivo. The maturation of DCs and selective modulation of cytokine production by CT to induce a DC2 type cell, in part through the activation of ERK MAP kinase, has also been discussed. However, the possibility that the immunomodulatory properties of CT also involved direct interaction with and modulation of T cell responses has to be considered.

The studies in DCs showed that the induction of ERK and subsequently c-fos appeared to be dependent on the enzymatic activity of the toxin. However, previous studies have shown that enzymatically inactive mutants of CT (Yamamoto et al., 1997a; Yamamoto et al., 1997b), as well as purified recombinant CTB (Jertborn et al., 2001; Tochikubo et al., 1998) retain adjuvant properties. Therefore, the adjuvanticity of CT must be dependent on the ability of the toxin to bind to its ligand, GM1, expressed on the surface of most nucleated cells. Interaction of CTB with GM1 ganglioside has recently been used as a method to detect lipid microdomains on T cells, as GM1 is a major component of lipid rafts (Ebert et al., 2000). More importantly however, stimulation of T cells with low α-CD3 monoclonal antibody concentration was enhanced by simultaneous treatment with CTB, suggesting that coaggregation of the lipid raft-associated protein, GM1 provided costimulation (Viola et al., 1999). This indicated that CT can directly interact with T cells and therefore suggests an additional mechanism for the adjuvanticity of the toxin.

The A subunit of CT may also contribute to the direct modulation of T cells through the enhancement of intracellular levels of cAMP. Many studies have
examined the impact of cAMP-elevating agents on T helper cell function and development (Benbernou et al., 1997; Gilmore and Weiner, 1988; Lacour et al., 1994). It has been suggested that increasing the cAMP levels by cAMP analogues, cAMP-elevating agents or PGE$_2$ results in inhibition of both T-cell proliferation and production of Th1-associated cytokines, but stimulation of Th2-associated gene expression (Gajewski et al., 1990; Munoz et al., 1990). However, the role of CT in the modulation of cytokine production by T cells and the involvement of the MAP kinase signalling family in this modulation is not fully understood.

Therefore, in this chapter the effect of CT on cytokine production from naïve T cells and established T cell clones was assessed. The data shows that CT inhibits anti-CD3-induced cytokine production and proliferation of naïve T cells and a Th1 clone, but enhances IL-5 production from an established Tr1 clone. CT also enhances Th2-associated cytokine production but inhibited IFN-γ production by mixed Th1/Th2 cell lines capable of producing both subtypes of cytokines. The findings also demonstrate a role for p38 and ERK in the direct induction of IL-5 production by CT the T cell line, EL4.IL-2.
5.2 Results

5.2.1 CT inhibits cytokine production and proliferation of anti-CD3-stimulated naïve CD4⁺ T cells

To investigate the effect of CT on naïve T cell responses, CD4⁺ T cell were isolated from the spleens of naïve BALB/c mice and stimulated with immobilized anti-CD3 in the presence or absence of CT. Unstimulated T cells or T cells incubated with CT alone did not produce detectable levels of any of the cytokines measured (Fig. 5.1A). In contrast, incubation of naïve T cells with immobilized anti-CD3, which engages the T cell receptor (TCR), resulted in the activation of naïve CD4⁺ T cells, which was characterised by the production of high levels of IFN-γ and IL-2, lower levels of IL-4 and IL-10 and barely detectable levels of IL-5 in the supernatants 48 hours later. However, addition of CT resulted in complete inhibition of cytokine production by naïve, anti-CD3-stimulated CD4⁺ T cells. Anti-CD3 stimulation resulted in a high rate of proliferation of these cells, which was completely abolished in the presence of CT, confirming the suppressive effect of CT on naïve T cell function (Fig. 5.1B). This data demonstrated that CT suppresses anti-CD3 stimulation of naïve CD4⁺ T cells.

5.2.2 The effect of CT on cytokine production from established T cell lines and clones

Having demonstrated that CT could modulate the activation of naïve T cells, I then assessed the ability of CT to modulate differentiated Ag-specific T cells. Established Ag-specific T cell lines or clones were employed and were stimulated with anti-CD3 or specific Ag. The cell lines and clones that were used were a influenza virus HA-specific Th1 clone and a FHA-specific Tr1 clone (described in
Figure 5.1  CT inhibits cytokine production and proliferation of α-CD3 stimulated naïve CD4⁺ T cells

Naïve CD4⁺ T cells were isolated through negative selection from BALB/c spleen cells using a mouse T cell CD4 subset column kit. T cells were stimulated with plate-bound anti-CD3 (10 μg/ml) in the presence or absence of CT (100 ng/ml). (A) Supernatants were removed after 48 hours, replaced with medium and IL-2, IL-4, IL-5, IL-10 and IFN-γ levels were determined in the supernatant by ELISA. (B) Proliferation was determined after 72 hours of culture by measuring the rate of incorporation of ³H-thymidine for 6 hours at 37°C. Results represent the mean (± SD) of triplicate cultures and are representative of two independent experiments. *** p < 0.001.
(McGuirk et al., 2002), both generated and maintained by Dr. Peter McGuirk, a liver fluke homogenate (LFH)-specific Th1/Th2 line generated and maintained by Dr. Miriam Brady and a KLH-specific Th1/Th2 line.

5.2.2.1 CT inhibits IFN-γ and IL-2 production from a Th1 clone

The Th1 clone HDS2.6 specific for influenza virus HA, which was generated from the spleens of mice infected with influenza viral Ags, has been shown previously to secrete IFN-γ and IL-2, but not IL-4, IL-5, IL-10 or TGF-β in response to antigenic stimulation (McGuirk et al., 2002). As expected, the Th1 clone produced the Th1-associated cytokines IFN-γ and IL-2 in response to anti-CD3 stimulation (Fig. 5.2). Addition of CT, however, resulted in the significant inhibition of both of these cytokines from the Th1 clone.

5.2.2.2 CT enhances IL-5 production from a Tr1 clone

The Tr1 clone, FHA1.1, which is specific for FHA, was generated from the lungs of mice after aerosol challenge with *B. pertussis*. Antigenic stimulation of this clone results in the production of IL-5 and IL-10, but very low levels of IFN-γ and undetectable IL-2 or IL-4 (McGuirk et al., 2002). The Tr1 clone, when stimulated with anti-CD3, produced low but detectable levels of IL-5 (Fig. 5.3). Co-incubation with CT resulted in the significant enhancement of IL-5 production from these cells. This data indicates that, in contrast to suppression of cytokine production from a Th1 clone, CT enhances IL-5 production from a Tr1 clone.
Figure 5.2  CT inhibits IFN-γ and IL-2 production from a Th1 clone

The Th1 clone HDS2.6, specific for influenza virus, was stimulated with plate-bound anti-CD3 (25 μg/ml) or medium only in the presence (+ CT) or absence (-CT) of CT (100 ng/ml) for 24 hours. IFN-γ and IL-2 concentrations were determined in the supernatant by ELISA. Results are mean (± SD) of triplicate cultures. *, p < 0.05; ***, p < 0.001 (with vs without CT). This work was done in conjunction with Rachel Clancy.
Figure 5.3  CT enhances IL-5 production from a Tr1 clone

The Tr1 clone FHA1.1, specific for FHA was stimulated with plate-bound anti-CD3 (25 μg/ml) or medium only in the presence (+ CT) or absence (-CT) of CT (100 ng/ml) for 24 hours. IL-5 concentrations were determined in the supernatant by ELISA. Results are mean (± SD) of triplicate cultures. *, p < 0.05 (with vs without CT). This work was done in conjunction with Rachel Clancy.
5.2.2.3 CT enhances Th2-associated cytokine production but inhibits IFN-γ from mixed Th1/Th2 cell lines

The effect on CT on cytokine production from an established T cell line capable of producing both Th1- and Th2-associated cytokines upon antigenic stimulation was assessed. An LFH-specific T cell line was generated from the hepatic LN of mice infected with *Fasciola hepatica*. The T cell line was incubated with irradiated APC and LFH as Ag in the presence or absence of CT. Stimulation of the Th1/Th2 cell line with APCs did not result in the production of detectable IL-4, IL-5, IL-10 or IL-13. However, IFN-γ was produced in the absence of Ag (LFH), suggesting that the T cell or APC are pre-activated for IFN-γ production (Fig. 5.4). Addition of Ag with the APC resulted in the production of high levels of IL-4, IL-5, IL-13 and IL-10 but no enhancement of IFN-γ, demonstrating that these T cells are capable of producing Th2-associated cytokines in response to antigenic stimulation. Co-incubation of Ag-activated T cells with 100 ng/ml of CT resulted in the significant enhancement of the Ag-induced Th2-type cytokines IL-4, IL-5, IL-10 and IL-13. In contrast, CT significantly inhibited production of the Th1-associated cytokine IFN-γ induced either in the presence or absence of Ag.

LFH is a parasite Ag and may possess its own modulatory properties, which could directly affect the induction of cytokine production from these T cells. Therefore, a T cell line specific for the model Ag KLH was used to verify the ability of CT to directly modulate T cell cytokine production. Following Ag stimulation the KLH-specific T cell line produced high levels of the Th2-associated cytokines IL-4, IL-5, IL-13 and IL-10 as well as the Th1-associated cytokine IFN-γ (Fig. 5.5). Addition of CT resulted in the significant enhancement of IL-4 and IL-5 production. IL-10 production was not affected by the presence of CT, while IL-13 and IFN-γ
Figure 5.4 CT enhances the Th2-associated cytokines IL-4, IL-5, IL-10 and IL-13 and inhibits IFN-γ production from an LFH-specific mixed Th1/Th2 cell line

The LFH-specific mixed Th1/Th2 cell line was generated from mice infected with *F. hepatica*. The established T cell line was stimulated with APCs in the presence or absence of Ag (LFH; 15 μg/ml), with (+ CT) or without (- CT) 100 ng/ml of CT. Supernatants were removed after three days and cytokine concentrations were determined by ELISA. Results represent the mean (± SD) of triplicate assay. **, p < 0.01; ***, p < 0.001 (with vs without CT).
Figure 5.5 CT enhances the Th2-associated cytokines IL-4 and IL-5 and inhibits IFN-γ and IL-13 production from a KLH-specific mixed Th1/Th2 cell line

The KLH-specific mixed Th1/Th2 cell line was generated from mice immunized with KLH and CT as adjuvant. The T cell line was stimulated with APCs in the presence or absence of Ag (KLH; 10 μg/ml), with (+ CT) or without (- CT) 100 ng/ml of CT. Supernatants were removed after three days and cytokine concentrations were determined by ELISA. Results represent the mean (± SD) of triplicate assay. **, p < 0.01; ***, p < 0.001; ns, not significant (with vs without CT).
production was significantly inhibited. These results indicate that CT can differentially modulate the production of Th1- and Th2-associated cytokines from Ag-specific T cells in vitro.

5.2.3 CT enhances PMA-induced IL-5 production while inhibiting IL-2, IL-4, IL-10 and IL-13 production from EL4-IL-2 cells

Having shown that CT could modulate cytokine production from T cells freshly isolated from spleen or from established T cell lines and clones, the modulatory effects of CT on the murine thymoma-derived T cell line EL4.IL-2 were assessed. In order to determine the most effective stimulus to use and the optimal length of stimulation, EL4.IL-2 cells were stimulated with anti-CD3 plus anti-CD28, PMA or PMA plus ionomycin over a time course from 3 to 54 hours (Fig. 5.6). Stimulation with anti-CD3 plus anti-CD28 resulted in the marginal activation of IL-2 and IL-4 production, in contrast to PMA or PMA plus ionomycin, which dramatically enhanced IL-2 and IL-4 as well as IL-5 and IL-10. The inclusion of ionomycin did not appear to enhance the stimulation of EL4.IL-2 cells by PMA, therefore PMA alone was used to stimulate these cells in the assessment of the modulation of cytokine production by CT.

EL4.IL-2 cells were incubated with increasing doses of PMA (0.78 - 50 ng/ml) in the presence or absence of CT for 24 hours (Fig. 5.7). PMA induced a dose dependent increase in IL-5 production from EL4.IL-2 cells. Addition of CT resulted in a lowering of the threshold for IL-5 induction by PMA from below 2 ng/ml to just over 0.5 ng/ml. A significant increase in IL-5 production in the presence of CT was observed at all of the doses of PMA used up to 12.5 ng/ml. The concentration of IL-5
Figure 5.6 Cytokine production by EL4.IL-2 cells in response to a number of stimuli over a time course

EL4.IL-2 cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (10 μg/ml), PMA (50 ng/ml) or PMA and ionomycin (1 μg/ml) for the indicated times. IL-5, IL-4, IL-10 and IL-2 levels were detected in the supernatant by ELISA. Results are mean (± SD) from triplicate assays.
Figure 5.7 CT significantly enhances PMA-induced IL-5 while inhibiting IL-4, IL-10, IL-13 and IL-2 from EL4.IL-2 T cells

EL4.IL-2 cells were stimulated with a series of doses of PMA (0.78 – 50 ng/ml) in the presence (CT (□)) or absence (control (□)) of CT (100 ng/ml) for 24 hours. IL-5, IL-4, IL-10, IL-13 and IL-2 concentrations were detected in the supernatant by ELISA. Results are mean (± SD) from triplicate assays and are representative of two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant (PMA vs PMA + CT).
carried out using EL4.IL-2 cells. Therefore, the effect of CT on IFN-γ production could not be assessed using these cells.

The results show that CT significantly enhanced PMA-induced IL-5 production, while significantly inhibiting the production of IL-4, IL-10, IL-13 and IL-2 from EL4.IL-2 cells.

5.2.4 CT directly induces the production of IL-5 from EL4.IL-2 cells

Having demonstrated that CT could enhance PMA-induced IL-5 production, the direct induction of this cytokine by CT was assessed by stimulating EL4.IL-2 cells with increasing doses of CT for 24 hours. Incubation of EL4.IL-2 cells with CT induced the production of significant levels of IL-5, with concentrations as low as 1 ng/ml of CT inducing detectable but not significant concentrations of IL-5 (Fig. 5.8A). A dose dependent increase in IL-5 was observed with increasing doses of CT, up to 1 μg/ml, where maximal concentrations were maintained. A time course experiment demonstrated the presence of detectable levels of IL-5 12 hours after stimulation with 100 ng/ml of CT. The levels of IL-5 continued to increase over time with high levels detected 48 hours after stimulation with CT (Fig. 5.8B). In agreement with IL-5 protein induction, IL-5 mRNA expression was strongly upregulated 12 hours after stimulation with CT compared with medium control and was still elevated after 48 hours (Fig. 5.9). The induction of IL-2, IL-4, IL-10 or IL-13 by CT was not observed from EL4.IL-2 cells over the course of these experiments.

5.2.5 Analysis of the role of the MAP kinases in CT-induced IL-5 production

Recent studies have addressed the role the MAP kinase signalling family in the differentiation of Th1 and Th2 cells, including their role in the induction of a
Figure 5.8  CT directly induces IL-5 production from EL4.IL-2 cells

(A) EL4.IL-2 cells were stimulated with increasing doses of CT (0.1–10,000 ng/ml) for 24 hours. (B) EL4.IL-2 cells were incubated for 12, 24 or 48 hours with (+CT) or without (-CT) 100 ng/ml of CT. Supernatants were analysed for IL-5 by ELISA. Results are the mean (± SD) of triplicate assays and are representative of three experiments. *, p < 0.05; ***, p < 0.001 (-CT vs +CT).
Figure 5.9 CT induces IL-5 mRNA expression in EL4.IL-2 cells

EL4.IL-2 cells were incubated with medium or with CT (100 ng/ml) for 12, 24 or 48 hours. IL-5 mRNA was determined by RT-PCR. In the upper panel IL-5 mRNA is shown. In the lower panel the β-actin mRNA shows that comparable amounts of product were amplified for each condition. Results represent PCR products from three replicates for IL-5 mRNA compared with β-actin.
variety of cytokines. Of particular interest is the identification of p38 as a critical factor in cAMP-induced IL-5 production (Chen et al., 2000). Therefore, I examined the potential involvement of members of the MAP kinase signalling family in the induction of IL-5 by CT.

5.2.5.1 CT induces the phosphorylation of p38 and ERK in EL4.IL-2 cells

EL4.IL-2 cells were stimulated with CT (100 ng/ml) for 90 mins and the phosphorylation of ERK and p38 were assessed by western blotting. Resting EL4.IL-2 cells expressed low levels of phosphorylated ERK (Fig. 5.10A) and undetectable levels of phosphorylated p38 (Fig. 5.10B). Stimulation of cells with CT enhanced the expression of both phosphorylated ERK and p38. This increase is not due to different levels of MAP kinase in the samples as indicated by similar levels of total ERK or total p38 (Fig. 5.10, lower panels). ERK consists of two isoforms, ERK1 and ERK2, of 44kDa and 42kDa, respectively. However, only the phosphorylated form of ERK2 (42kDa) was detected in EL4.IL-2 cells in all of the experiments carried out, although both ERK1 and ERK2 were present in the cells, as indicated with antibodies specific for total ERK. Therefore, CT induces the activation of p38 and ERK1 in the T cell line EL4.IL-2.

5.2.5.2 The p38 inhibitor SB203580 inhibits CT-induced IL-5 production

The role of p38 in CT-induced IL-5 was assessed by pre-incubating EL4.IL-2 cells with increasing doses of the p38 specific inhibitor, SB203580, followed by stimulation with CT. EL4.IL-2 cells did not production IL-5 production in the absence of CT stimulation (Fig. 5.11). As previously described, stimulation with CT (100 ng/ml) induced significant levels of IL-5. CT-induced IL-5 production was
Figure 5.10  CT induces the phosphorylation of ERK and p38 in EL4.IL-2 cells
EL4.IL-2 cells were stimulated with CT (100 ng/ml) for 90 mins. Cells were lysed in
100 μl of 1x sample buffer, sonicated, boiled and separated by 12% SDS-PAGE.
Samples were transferred to nitrocellulose and incubated with Abs specific for
phospho-ERK (A) or phospho-p38 (B). The nitrocellulose was then stripped and
assayed for ERK1 (A) or p38 (B). The upper panel of A or B shows the
phosphorylated form of each kinase, while the lower panel shows total kinase levels.
Identical results were obtained in three further experiments.
Figure 5.11 The p38 MAPK inhibitor SB203580 inhibits CT-induced IL-5 production from EL4.IL-2 cells

EL4.IL-2 cells were incubated with medium or increasing doses of the p38 inhibitor SB203580 (0.1 - 1 μM) for 1 hour before addition of CT (100 ng/ml) for 24 hours. IL-5 concentrations were determined in the supernatant by ELISA. Results are the mean (± SD) of triplicate assays and are representative of three experiments. ***, p < 0.001 (untreated vs CT); ###, p < 0.001 (CT vs CT + inhibitor); ns; not significant (untreated vs CT + inhibitor).
significantly inhibited, in a dose dependent manner, by SB203580, a specific inhibitor for p38 activity. Concentrations of SB203580 as low as 0.1 μM significantly inhibited CT-induced IL-5. The concentration of IL-5 induced by CT in the presence of the p38 inhibitor (1 μM) was not significantly higher than that detected in unstimulated cells. These results suggest that CT-induced IL-5 production involves the activation of the MAP kinase p38.

5.2.5.3 ERK is involved in CT-induced IL-5 production

To examine the role of the MAP kinase ERK in the induction of IL-5 by CT in EL4.IL-2 cells, two MEK1/2-specific inhibitors were utilized, PD98059 and U0126. CT induced IL-5 production from EL4.IL-2 cells was completely abrogated in the presence of U0126 (Fig. 5.12A). Similarly, incubation of EL-4.IL-2 cells with increasing doses of PD98059 one hour prior to stimulation with CT resulted in the dose dependent inhibition of IL-5 production (Fig. 5.12B). Complete inhibition of CT-induced IL-5 production was observed with a 10 μM concentration of the ERK inhibitor PD98059. This data illustrates a requirement for the MAP kinase ERK in CT-induced IL-5 production in EL4.IL-2 cells.

5.2.5.4 The JNK inhibitor SP600125 enhances CT-induced IL-5 production from EL4.IL-2 cells.

The role of the MAP kinase JNK in CT-induced IL-5 was assessed using the JNK inhibitor, SP600125. Incubation of EL4.IL-2 cells with the JNK inhibitor prior to stimulation with CT resulted in the significant augmentation of IL-5 production (Fig. 5.13). Enhanced CT-induced IL-5 production was observed with increasing
Figure 5.12  The ERK pathway inhibitors (A) U0126 and (B) PD98059 inhibit CT-induced IL-5 production from EL4.IL-2 cells

EL4.IL-2 cells were incubated with medium or increasing doses of the MEK inhibitors (A) U0126 (0.5 μM – 2 μM) or (B) PD98059 (1 – 10 μM) for 1 hour before the addition of CT (100 ng/ml) for 24 hours. IL-5 concentrations were determined in the supernatant by ELISA. Results are the mean (± SD) of triplicate assays and are representative of three experiments. ***, p < 0.001 (untreated vs CT); ###, p < 0.001 (CT vs CT + inhibitor).
Figure 5.13 The JNK inhibitor SP600125 enhances CT-induced IL-5 production from EL4.IL-2 cells

EL4.IL-2 cells were incubated with medium or increasing doses of the JNK inhibitor SP600125 (2.5–10 μM) for 1 hour before addition of CT (100 ng/ml) for 24 hours. IL-5 concentrations were determined in the supernatant by ELISA. Results are the mean (± SD) of triplicate assays and are representative of three experiments. **, p < 0.01 (untreated vs CT); ###, p < 0.001 (CT vs CT + inhibitor).
doses of the JNK inhibitor, up to 10 µM. Therefore, unlike p38 and ERK, the JNK MAP kinase provides an inhibitory signal for IL-5 production in EL4.IL-2 cells.

5.2.6 The role of the MAP kinase family in PMA-induced IL-5

In order to determine if the effects of the MAP kinase inhibitors were specific for CT-induced IL-5 production or whether the MAP kinases play a similar role in IL-5 production induced by other stimuli, EL4.IL-2 cells were pre-incubated with the inhibitors at a variety of doses for 1h followed by stimulation with PMA for 24h. As previously demonstrated, stimulation of EL4.IL-2 cells with PMA results in the induction of high levels of IL-5. Pre-incubation with the p38 inhibitor, SB203580 significantly inhibited PMA-induced IL-5 production in EL4.IL-2 cells (Fig. 5.14). A similar trend was observed with the ERK inhibitors, U0126 and PD98059. Increasing doses of each of these inhibitors significantly reduced IL-5 production by PMA-stimulated EL4.IL-2 cells with U0126 at 8 µM completely inhibiting IL-5 production. Pre-incubation of PMA-stimulated EL4.IL-2 cells with the JNK inhibitor SP600125 resulted in a significant increase in IL-5 production. These data indicate a similar role for the MAP kinase signalling family in PMA-induced IL-5 production to that observed with CT-induced IL-5 production by EL4.IL-2 cells. Therefore, both p38 and ERK appear to be essential for IL-5 production, whereas the JNK pathway provides an inhibitory signal for the secretion of IL-5.

5.2.7 The role of GATA-3 in CT-induced IL-5 production

GATA-3, a transcription factor that is critical for thymocyte development (Ting et al., 1996) is expressed at a high level in naïve CD4⁺ T cells and Th2 cells, but at a low level in Th1 cells (Zheng and Flavell, 1997). A critical role for this
Figure 5.14 Effect of the p38 inhibitor SB203580, the MEK1/2 inhibitors U0126 and PD98059 or the JNK inhibitor SP600125 on PMA-induced IL-5 production from EL4.IL-2 cells

EL4.IL-2 cells were incubated with medium or increasing doses of the p38 inhibitor SB203580 (0.1 – 1 μM), the MEK1/2 inhibitors U0126 (2 – 8 μM) or PD98059 (5 – 25 μM) or the JNK inhibitor SP600125 (5 – 25 μM) for 1 hour before addition of PMA (50 ng/ml) for 24 hours. IL-5 concentrations were determined in the supernatant by ELISA. Results are the mean (± SD) of triplicate assays and are representative of two experiments. *, p < 0.05; **, p < 0.01, ***, p < 0.001 (PMA vs PMA + inhibitor).
transcription factor in Th2 cell development and specifically in the expression of IL-5 in Th2 cells has been described (Zhang et al., 1997). Therefore, the level of GATA-3 in resting and CT stimulated EL4.IL-2 cells was measured using two different methods.

GATA-3 gene expression was analysed by RT-PCR in EL4.IL-2 cells stimulated with CT from 12 to 48 hours (Fig. 5.15). A basal level of GATA-3 mRNA expression was detected in EL4.IL-2 cells that were cultured with medium only at all of the time points examined. Stimulation with CT (100 ng/ml) did not affect GATA-3 gene transcription in EL4.IL-2 cells at any of the time points examined. β-actin PCR is shown as a control of the relative amount of cDNA loaded in the different lanes.

Transcription factors can be upregulated rapidly in response to a stimulus; therefore CT may have enhanced GATA-3 expression, which subsequently returned to basal levels before the 12 hour time point. To examine this possibility, GATA-3 expression in EL4.IL-2 cells stimulated for 1, 2 or 3 hours with CT was analysed by western blotting (Fig. 5.16). GATA-3 protein was abundantly expressed in resting T cells and addition of CT did not appear to alter these concentrations. The bottom panel shows β-actin levels in the cells, demonstrating equal amounts of protein in each sample. This data indicates that EL4.IL-2 cells endogenously express the transcription factor GATA-3, which is not modulated by CT.
Figure 5.15  CT does not enhance GATA-3 mRNA expression in EL4.IL-2 cells
EL4.IL-2 cells were incubated with medium or CT (100 ng/ml) for 12, 24 or 48 hours. GATA-3 mRNA was determined by RT-PCR. β-actin PCR is shown as a control to show equal loading of cDNA in the different lanes. Results are representative of PCR products from three replicates for IL-5 mRNA compared with β-actin.
Figure 5.16  **CT does not enhance early GATA-3 expression in EL4.IL-2 cells**  
EL4.IL-2 cells were incubated with medium or CT (100 ng/ml) for 1, 2 or 3 hours. Cells were lysed in 100 µl of 1 x sample buffer, sonicated and boiled. Samples were separated by 12% SDS-PAGE, transferred to nitrocellulose and assayed for GATA-3 by immunoblotting of lysates. The nitrocellulose was then stripped and assayed for β-actin. The upper panel shows GATA-3 while the lower panel shows β-actin, indicating equal loading of samples. Identical results were obtained in two further experiments.
In this study the direct modulation of T cell cytokine production by CT was investigated. The results demonstrate that CT enhances Th2-associated cytokine production but inhibits Th1-associated cytokines. In addition, CT directly induces the production of the Th2-associated cytokine IL-5 from the thymoma T cell line EL4.IL-2 in a p38- and ERK-dependent fashion.

The data presented in this chapter demonstrates that CT directly suppresses the production of IL-2, IFN-γ and proliferation of naïve CD4⁺ T cells, in addition to suppressing IFN-γ and IL-2 from a Th1 clone in response to activation via the TCR-CD3 complex. In addition, the results show that CT inhibits IFN-γ production from two distinct Ag-specific mixed T cell lines and IL-2 production from PMA-stimulated EL4.IL-2 cells. These observations are in agreement with a number of previous reports, which show that CT inhibits the production of Th1-associated cytokine production (Aussel et al., 1988; Munoz et al., 1990; Yamamoto et al., 1999). The ability of CT to suppress Th1-associated cytokine responses appears to involve the enhancement of intracellular cAMP levels; similar results have been shown using a number of different cAMP-elevating agents (Benbernou et al., 1997; Gilmore and Weiner, 1988; Munoz et al., 1990), which indicate an inhibitory role for cAMP in Th1 cell development.

The induction of both IFN-γ and IL-2 appears to require three different transcription factor families, NFAT, NFκB and AP-1. It has been suggested that the potent transcriptional repressor ICER, which is stimulated by cAMP, is involved in the inhibition of NFAT- and AP-1-dependent genes, such as IL-2 and IFN-γ (Bodor and Habener, 1998). ICER can interact with the NFAT/AP-1 composite DNA site(s) in the promoter region, thereby preventing transcription factor interaction and
transcription of these genes. Interestingly, our group has demonstrated that CT induces ICER mRNA expression in EL4.IL-2 cells (M.A. Armstrong, unpublished observations). This is a possible mechanism by which CT suppresses Th1-associated cytokine production. In addition, cAMP-induced PKA activation was shown to inhibit in vitro DNA binding activities of NFκB in EL4.E1 cells (Chen and Rothenberg, 1994), thereby suggesting an additional cAMP-dependent mechanism for the inhibition of Th1 cytokine secretion through disruption of NFκB function. However, the suppressive effect of CT may not be entirely dependent on cAMP. An enzymatically inactive CT mutant, CTE112K, has been shown to suppress T cell proliferation and IFN-γ production from anti-CD3 stimulated T cells, without modulating the production of IL-4 (Yamamoto et al., 1999), which suggests that, in addition to enhancing cAMP, CT may utilise an alternative, undefined, mechanism to suppress Th1 cell activation.

In contrast to the suppressive effect of CT on Th1-associated cytokine production, the present data demonstrate that CT can enhance the production of the Th2-associated cytokines IL-4 and IL-5 by Ag-specific T cell lines (Fig. 5.4 and 5.5). However, CT inhibited the production of IL-4, IL-10 and IL-13 from anti-CD3 stimulated naïve CD4+ T cells and from PMA-stimulated EL4.IL-2 cells. The inhibition of IL-4 and IL-10 from PMA-stimulated EL4 cells by the cAMP elevating agent Bt2cAMP has previously been described (Lee et al., 1993). The mechanism behind the induction of Th2-associated cytokines from one cell population and not from others is not known, but may reflect the different expression of certain transcription factors in different cell populations. Also, the additional activation stimulus used (e.g. anti-CD3 or PMA versus Ag-specific stimulation) may play a role in the contrasting responses observed. However, the enhancement of cytokine
production from Ag-specific T cells may be the most accurate representation of the action of CT in vivo, where CT could directly interact with T cells primed by DCs previously stimulated with the toxin.

It is not known whether the enhancement of Th2-associated cytokines from mixed Th1/Th2 Ag-specific cell lines in this study is dependent on enhanced cAMP production. A previous examination of the effect of CT on a Th2 cell line showed no modulation of IL-4 production (Munoz et al., 1990). In addition, elevation of cAMP concentrations did not significantly modulate the concentration of IL-4 induced by CD4^+ T cells stimulated with anti-CD3 and anti-CD28 (Ozegbe et al., 2004) or from PHA and PMA stimulated Jurkat T cells (Benbernou et al., 1997). However, in agreement with the results presented here, CT or the cAMP elevating agent, forskolin, enhanced IL-4 production in activated CD4^+ T cells, implicating a role for cAMP in the enhancement of this cytokine (Lacour et al., 1994). These differences in the affect of cAMP on IL-4 production are consistent with the observation in the present study, which show that CT can have a different effect on the same cytokine in different cell types. The modulation of IL-13 by CT in this study was somewhat inconsistent. However, the overall observation is that CT suppressed IL-13 production. A previous study showed that Bt2cAMP did not suppress or enhance PMA/PHA-induced IL-13 from Jurkat cells (Benbernou et al., 1997), therefore the mechanism behind the modulation of IL-13 by CT remains to be defined. Elevated cAMP levels may play a role in the enhancement of IL-10 production as the cAMP elevating agent PGE2 has been shown to upregulate IL-10 mRNA expression in PHA and PMA stimulated Jurkat T cells (Benbernou et al., 1997).

In contrast to the inconsistent modulation of the other Th2-associated cytokines, CT enhanced the production of IL-5 from the thymoma cell line EL4.IL-2,
in addition to the Ag-specific T cell lines and from a Tr1 clone. In agreement with these findings, a previous study demonstrated that cAMP can enhance IL-5 production from PMA/ionomycin-stimulated CD4+ T cells (Lacour et al., 1994). The enhancement of IL-5 observed in vitro from primed T cells is consistent with the induction of Ag-specific IL-5 producing cells by CT in vivo and suggests that the adjuvant effect of CT may involve direct activation of T cells as well as APCs (chapter 3; Lavelle et al., 2003). However, reports in the literature are not all in agreement with the observed enhancement of IL-5 in this study. Stimulation of PBMCs with anti-CD3 and anti-CD28 in the presence of CT or cAMP-elevating agents was shown to inhibit IL-5 production (Staples et al., 2001). However, IL-5 production from PMA/PHA-treated PBMC was completely unaffected by cAMP-elevating agents (Staples et al., 2000). IL-5 production from type II collagen-specific T cells was slightly increased in the presence of low doses of cAMP-elevating agents, but was modestly inhibited at higher doses (Ozegbe et al., 2004). Taken together, these results indicate that the type of T cell, the doses of the modulatory agent and the additional stimulatory signal used may affect the ability of CT to modulate IL-5 production.

In the present study, not only did CT synergise with PMA to induce IL-5 in EL4.IL-2 cells, it directly induced the production of this cytokine in the absence of any other activating signal. Previous reports have demonstrated that cAMP could enhance IL-5 production from EL4 cells (Karlen et al., 1996; Lee et al., 1993; Lee et al., 1995); however, an additional stimulatory signal such as PMA was required. Therefore, since CT directly promotes IL-5 production, it appears that this toxin may induce an additional signal to cAMP, which is necessary for the induction of IL-5 in EL4 cells.
GATA-3 is a multifunctional transcription factor that is essential for T cell development and Th differentiation. It is expressed at a high level in naïve CD4+ T cells, and this expression is maintained in Th2 polarizing cells, but is rapidly downregulated during Th1 commitment (Ferber et al., 1999; Zheng and Flavell, 1997). Ectopic expression of GATA-3 in developing Th1 cells gives rise to Th2-associated cytokine production (Ouyang et al., 1998). However, GATA-3 does not directly bind to the IL-4 or IL-13 gene promoter (Schwenger et al., 2001; Zhang et al., 1999). The genes encoding IL-4, IL-5 and IL-13 are clustered on chromosome 11 in mice and work carried out by Lee and colleagues has shown that the positive action of GATA-3 on Th2 cytokine production is mediated through its effects on the entire Th2 locus, probably through chromatin remodelling, during Th2 cell differentiation (Lee et al., 2003). Much of the work to date on the induction of IL-5 gene transcription has been carried out in the EL-4 T cell line and has shown that activation of the IL-5 promoter requires at least two DNA elements, a double GATA site and an AP-1 site within a CLEO (conserved lymphokine element O) (Zhang et al., 1998). The GATA site in the IL-5 promoter binds the transcription factor GATA-3, but not GATA-4 and GATA-3, and is necessary, but not sufficient, for IL-5 gene transcription (Siegel et al., 1995). Therefore, the differential requirement of GATA-3 in IL-5 and IL-4 production may provide the basis for independent expression of these two cytokines.

This study has shown that GATA-3 is expressed at a high level in resting EL4.IL-2 cells. However, CT did not directly upregulate the expression of GATA-3 in this cell line. Therefore, it appears that the induction of IL-5 by CT is independent of the upregulation of GATA-3 expression by the toxin in EL4.IL-2 cells. There are two possible explanations as to why the IL-5 gene is not constitutively expressed in unstimulated EL4 cells, despite high levels of GATA-3. Firstly, GATA-3 protein
may require posttranslational modification or activation for optimal function. Secondly, binding of proteins to the AP-1 site within the CLEO in the IL-5 promoter is also required for IL-5 transcription, which is achieved only when the cells are activated. These observations may also suggest a possible mechanism by which CT directly induces IL-5 production in EL4.IL-2 cells. In addition, analysis of the IL-5 promoter region revealed a number of binding sites for several transcription factors, but no CRE (Lee et al., 1995), which would indicate an alternative mechanism(s) of gene regulation for IL-5 production by CT, independent of the cAMP-dependent PKA/CREB pathway (which requires a CRE element). Interestingly, PKA has been shown to be involved in cAMP-enhanced IL-5 production in a GATA-3-dependent manner (Klein-Hessling et al., 2003) and therefore may function through GATA-3 modification. Consistent with this suggestion is the observation that there are several potential PKA, as well as PKC phosphorylation sites, in the GATA-3 protein (Zhang et al., 1997), thereby providing a mechanism by which PKA may be involved in IL-5 production.

Data presented in this study shows that CT directly induces the phosphorylation of p38 in EL4.IL-2 cells and that this kinase is essential for IL-5 production by CT. Inhibition of p38 in PMA-stimulated EL4.IL-2 cells reduced the production of IL-5, confirming the role of this kinase in IL-5 production. The p38 MAP kinase has previously been associated with cAMP-enhanced IL-5 and IL-13 production (Chen et al., 2000). Direct stimulation of the cAMP pathway with bt2cAMP induced p38 phosphorylation in Th2 D10 cells but not in Th1 cells. Inhibition of p38 phosphorylation resulted in suppression of PMA/bt2cAMP-induced IL-5 production. The affect of cAMP-induced p38 on IL-5 production was shown to involve the phosphorylation of GATA-3 (Chen et al., 2000). It is possible therefore,
that the mechanism of CT-induced IL-5 production may involve the phosphorylation of GATA-3 in a cAMP/p38 dependent manner; however, this phosphorylation of GATA-3 by CT has yet to be demonstrated.

Data presented in this study show a critical role for another member of the MAP kinase signalling family, ERK, in CT-induced IL-5 production. This study shows, for the first time, the induction of ERK phosphorylation by CT in T cells, with inhibition of ERK resulting in the complete inhibition of CT-induced IL-5 production. ERK, through TCR-mediated activation, has been shown to be involved in IL-4R function and to be required for Th2 differentiation in vivo and in vitro (Yamashita et al., 1999). The present study shows a positive role for ERK in IL-5 production, which is in agreement with a previous study that demonstrated a role for ERK in the optimal production of IL-5 from anti-CD3/anti-CD28 stimulated, freshly isolated T lymphocytes (Heijink et al., 2002).

An examination of the CLEO region of the murine IL-5 promoter in EL4 cells showed that the inducible complex that binds this element is composed of c-fos and JunB (Karlen et al., 1996; Siegel et al., 1995). As previously discussed, ERK is an upstream kinase for c-fos activation (Gille et al., 1992), thereby presenting a potential link between ERK activation by CT and the induction of IL-5 gene transcription. Interestingly, p38 activity has been shown to be required for the induction of JunB expression in primary murine T cells and that JunB is phosphorylated by p38 MAPK (Zhang et al., 2001). Therefore, the role of p38 in the induction of IL-5 by CT may also involve the induction and activation of JunB, part of the AP-1 complex necessary for IL-5 gene transcription.

Inhibition of JNK resulted in the enhancement of IL-5 in response to CT, indicating a negative role for this kinase in IL-5 production in T cells. This is
consistent with previous reports which show that CD4^+ T cells from JNK1 deficient mice selectively differentiate to become Th2 effector cells \textit{in vitro} (Yang et al., 1998). Furthermore, JNK1 deficient mice display an enhanced Th2 response against \textit{Leishmania major} (Constant et al., 2000), indicating a role for JNK1 as a negative regulator of Th2 cell development.

In conclusion, CT promotes Th2 cell activation by inhibiting the production of Th1-promoting factors, while directly enhancing Th2-associated cytokines. It appears that upregulation of cAMP by CT may play an important role in the modulation of T cell activation and possibly their differentiation and that different Th cell subsets appear to differ in their sensitivity to CT. In addition, the ability of CT to directly induce IL-5 production in a p38 and ERK dependent manner may contribute to the adjuvant effect of this toxin for the induction of Th2/Tr1 type immune responses \textit{in vivo}. 
Chapter Six

Final Discussion
Chapter 6

General Discussion

The development of new vaccines, including those that are effective by the mucosal route and those that selectively enhance one arm of the immune response, are dependent on the identification of new adjuvants and on defining their mode of action. Certain adjuvants have the ability to polarise T cell responses to distinct subtypes that may be of importance in protective immunity to individual pathogens. AB bacterial toxins, such as CT and LT, are probably the most powerful and best studied mucosal adjuvants to date (Rappuoli et al., 1999). However, a number of recipients of a nasal influenza vaccine that included wild-type LT as adjuvant developed Bell’s palsy (paralysis of the facial nerve) and this potential toxicity has led to the withdrawal of this vaccine from the market (Gluck et al., 1999; Mutsch et al., 2004). In addition, CT has been shown to traffic to the brain via the olfactory nerve when given intranasally in mice (van Ginkel et al., 2000). Therefore, these molecules are unlikely to be acceptable for widespread use in human vaccine preparations. However, CT is an invaluable tool in the examination of the events involved in the polarization of an immune response in response to bacterial products and its study will assist in the design of appropriate mucosal vaccines. Although much is known about the cytokines involved in T cell differentiation and the role of transcription factors in T cells during this process, little is known about the mechanisms involved in the polarization of distinct T cell subtypes. Therefore this study, through the utilization of the immunomodulatory molecule CT, examined some the factors involved in the modulation of DCs and T cells in the polarization of immune responses.

CT is a potent adjuvant and this study confirmed previous observations that CT enhances Th2/Tr1 biased responses to coadministered Ag, with enhanced Ag-
specific IL-4, IL-5, IL-10 and IL-13, but low IFN-γ production. We have previously demonstrated that CT can enhance, in addition to classic Th2 cells that produce IL-4 or IL-4 and IL-10, a population of IL-10-producing T cells with suppressor activity (Lavelle et al., 2004). This population of Ag-specific CD4+ Tr1 cells, generated from mice immunized with Ag in the presence of CT, produces IL-10 independently of IL-4 and suppresses proliferation and IFN-γ production by Th1 cells. This may represent a potent mechanism evolved by *V. cholerae* to subvert protective Th1 responses.

The mechanisms involved in the modulatory activities of CT and the role of different signalling cascades in the modulation of immune cells are not fully understood. DCs play a critical role in the modulation and differentiation of T cell responses through the diverse expression of co-stimulatory molecules on their surface and the varied cytokine profiles induced after interaction with a pathogen. Therefore, the initial investigations concerned the study of the effects of CT on the maturation and induction of cytokine production in DCs. The results demonstrate that CT directly promotes the maturation of DCs, with upregulation of CD80 and CD86 expression. These costimulatory molecules play an important role in the induction of the adaptive immune response through their interaction with CD28 on the surface of T cells, and therefore provide the necessary costimulatory signal for T cell proliferation. Upregulation of CD80 and CD86 has previously been associated with the adjuvanticity and immunogenicity of CT *in vivo* (Cong et al., 1997). In addition to their role in T cell activation, CD80 and CD86 may also be responsible for the selective augmentation of specific T cell subtypes (Elloso and Scott, 1999). However, the role of these costimulatory molecules in the polarization of Th cells in response to CT has not been determined. In addition to the direct enhancement of CD80 and CD86 expression, CT modulates the expression of other surface molecules induced by
TLR ligands, including the Th1-associated co-stimulatory molecule CD40. Suppression of this molecule by CT may play a role in the polarization of Trl/Th2 responses by CT. Indeed, ineffective CD40 signalling has been associated with T cell unresponsiveness and reduction in Th1-associated cytokine production, but enhanced IL-10 production (Martin et al., 2003).

In addition to the modulation of surface marker expression on LPS-stimulated DCs, this study also demonstrates that CT has the capacity to modulate chemokine and cytokine production. CT directly induced the production of MIP-2, an important chemokine for the recruitment of neutrophils, and which appears to be a mitogen for epithelial cells (Driscoll et al., 1995). However, the exact functional relevance of MIP-2 induction by CT in its adjuvant activity \textit{in vivo} is not yet known. A number of cytokines produced by cells of the innate immune system and/or T cells play an important role in the differentiation of Th cells to distinct subsets. IL-12 is a potent inducer of Th1-cell development, while IL-4, IL-6 and IL-10 have all been associated with Th2 cell differentiation (reviewed in Mosmann and Coffman, 1989). IL-10 and TGF-β may be involved in driving the differentiation of Tr1 cells (Chen and Rothenberg, 1994; McGuirk et al., 2002; Seder et al., 1998). This study demonstrates that CT is a potent inhibitor of IL-12 production from LPS- or CpG-stimulated DCs. In contrast to the inhibition of IL-12, CT enhances the production of the Th2-promoting cytokines, IL-1β and IL-10 from LPS-stimulated DCs. In addition, CT enhances LPS-induced IL-6 production (Lavelle et al., 2004). Enhanced IL-10 production from DCs has been associated with the generation of Tr1 cells by CT (Lavelle et al., 2003) (summarised in Fig. 6.1).

Data presented in this study also demonstrated an additional layer of regulation of the immune response by CT through the direct modulation of T cell
Figure 6.1 Proposed model of the modulation of innate and adaptive immune responses by CT. Through interaction with GM-1 ganglioside, CT enters the cell and enhances intracellular cAMP levels and ERK phosphorylation. CT selectively inhibits the expression of CD40 and ICAM, which are associated with Th1 cell development. In addition, CT suppresses the induction of the Th1-inducing cytokine IL-12, via a mechanism that involves, in part, ERK activation. In contrast, CT enhances the expression of other co-stimulatory molecules, including CD80/CD86, which are required for T cell activation, and in the presence of a second signal (e.g. LPS) enhance the production of IL-6 and IL-10 which are associated with Th2/Tr1 cell development. The enhancement of IL-10 involves the activation of ERK. CT also directly suppresses the production of IFN-γ from Th1 cells and enhances the production of IL-4, IL-5 and IL-10 from Th2 and Tr1 cells, thereby further promoting the development of a Th2/Tr1 response. TCR, T cell receptor; LFA-1, lymphocye function-associated antigen-1; ICAM-1, intracellular adhesion molecule; ERK, extracellular signal regulated kinase.
cytokine production. The results show that CT directly inhibits the production of the Th1-associated cytokines IFN-γ and IL-2 from anti-CD3 stimulated naïve T cells, and IFN-γ from a Th1 clone or mixed Th1/Th2 cell lines in vitro. This is consistent with the observations in this study that CT suppresses Ag-specific IFN-γ production induced by LPS in vivo. In addition to the direct suppression of cytokines from T cells, CT directly augmented Th2-associated cytokine production and IL-10 from mixed Th1/Th2 cell lines in vitro. This direct enhancement of Th2/Tr1-associated cytokines by CT from T cells may, in part, explain the preferential enhancement of IL-4, IL-5, IL-10 and IL-13 production by T cells specific for bystander Ag in vivo.

Many of the signalling pathways involved in CT-induced modulation of cytokine production from cells of the immune system are not known. Data present here demonstrates for the first time that CT induces phosphorylation of the MAP kinase ERK in DCs. Similar to other Th2 adjuvants, such as SEA, CT induced sustained activation of ERK signalling. The effect on ERK phosphorylation mirrors the sustained CT-induced activation of the immediate-early gene product c-fos in DCs. Indeed, CT-induced c-fos activation appears to be downstream of ERK, as inhibition of ERK with the specific inhibitor U0126, inhibited c-fos activation. The induction of ERK by CT appears to be functionally significant in its immunomodulatory activity. CT-induced MIP-2 production from DCs was dependent on ERK phosphorylation. Furthermore, the enhancement of CD80 in response to CT appears to involve, at least in part, the activation of ERK; in contrast, CD86 enhancement appears to be independent of this kinase.

Previous studies have demonstrated an important role for ERK activation as a negative regulator of IL-12 production (Feng et al., 1999; Puig-Kroger et al., 2001). Therefore, I proposed that inhibition of IL-12 by CT may involve activation of ERK.
in DCs. Indeed, CT is an effective inducer of ERK phosphorylation in DCs and inhibition of ERK enhanced IL-12 production in response to LPS from DCs. A recent study has suggested an important role of c-fos in the suppression of IL-12 production by enhanced ERK signalling (Dillon et al., 2004). Data presented here demonstrated that CT induced c-fos expression in DCs, therefore providing a possible role for this transcription factor in the suppression of IL-12 production by CT. However, suppression of ERK did not reverse CT-induced suppression of LPS-induced IL-12 production suggesting that an alternate signalling pathway may also be involved.

IL-10 is a potent regulatory cytokine with an anti-inflammatory role, potently inhibiting the capacity of APCs to produce inflammatory mediators, such as IL-12, TNF-α and IL-1 (Aste-Amezaga et al., 1998; Moore et al., 2001). However, little is known about the signalling pathways involved in its production by DCs. This study demonstrated an essential role for p38 MAP kinase in IL-10 induction in response to TLR4-signalling. This is in agreement with a previous study which showed that LPS activation of p38 MAP kinase is required for the production of IL-10 from macrophages (Salmon et al., 2001). However, it appears that maximal production of IL-10 requires an additional stimulus (Edwards et al., 2002). The present study demonstrated that p38 and ERK phosphorylation are involved in CT induced IL-10 production. I propose that LPS induces p38 activation, which induces IL-10 production, but that the additional, prolonged activation of ERK by CT results in the potent synergistic effect of CT and LPS for IL-10 production. Indeed, a role for c-fos, as a downstream effector of prolonged ERK activation may contribute to IL-10 production, as stimulation of DCs from c-fos<sup>−/−</sup> mice via TLR2 has been shown to result in impaired IL-10 production (Dillon et al., 2004). Therefore, prolonged activation of ERK may represent a mechanism by which CT can direct a DC to
promote Th2/Tr1 cell development through the suppression of IL-12 and enhancement of IL-10 production. Indeed, activation of c-fos in DCs in response to microbial signals is now emerging as an important pathway in the development of DCs that promote Th2 responses (Pulendran, 2004). Classic Th2 stimuli such as SEA, induce high concentrations of IL-12p70 from DCs which are devoid of the transcription factor c-fos (Agrawal et al., 2003) (summarised in Fig. 6.2).

In addition to enhancing Th2/Tr1-associated cytokine production from Ag-specific T cell lines, CT directly induces the production of the Th2-associated cytokine IL-5 from the T cell line EL4.IL-2. An examination of the activation of members of the MAP kinase family demonstrated that CT directly induces the activation of both p38 and ERK in EL4.IL-2. Inhibition of p38 or ERK resulted in the inhibition of CT-induced IL-5 production, illustrating the important role of the MAP kinase family in the induction of IL-5. The phosphorylation of p38 has previously been associated with IL-5 production in T cells and may involve the transcription factor GATA-3 (Chen et al., 2000). ERK has also been shown to play an important role in IL-5 and IL-10 production in response to TCR stimulation; however, this is the first demonstration of a role for MAP kinases in the modulation of T cells by CT. These observations demonstrate that the immunomodulatory effects of CT involve activation of the MAP kinase signalling family in different cells of the immune system.
Figure 6.2 Summary of the role of MAP kinase in the modulation of DCs by CT

CT, which binds to GM1 on the surface of the cell, induces ERK phosphorylation in DCs in a cAMP-dependent manner. ERK activation plays a role in the upregulation of CD80 but not CD86 on the surface of the cells and is essential for MIP-2 expression in response to CT. IL-10 production required p38 phosphorylation, but ERK activation is involved in maximal production of this cytokine. CT-induced ERK is also involved in the suppression of IL-12; however, additional mechanisms are also involved. Finally, CT induces c-fos activation, which may play a role in the modulation of IL-10 and IL-12 (dotted line).

Abbreviations used: CT, cholera toxin; TLR4, toll-like receptor 4; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein.
Chapter 7

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