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ACTIVATION OF INNATE IMMUNITY BY *BORDETELLA PERTUSSIS*

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Sarah Higgins

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ABSTRACT

Respiratory infection with *Bordetella pertussis* is associated with the induction of Th1 cells and these cells, as well as antibody and cells of the innate immune system mediate the clearance of bacteria from the lungs. Signalling through toll-like receptors (TLR) activates dendritic cell (DC) maturation and IL-12 production, which directs the induction of Th1 cells. In contrast, DC activated by other pathogen-derived molecules such as *B. pertussis* filamentous hemagglutinin and adenylate cyclase toxin enhance TLR ligand induced IL-10 production, which in turn, promotes the induction of type 1 regulatory T (Tr1) cells. This study found that the production of IL-10, in addition to inflammatory cytokines and chemokines, was significantly reduced in DC from TLR4-defective mice in response to *B. pertussis*. TLR4 was also required for *B. pertussis* LPS-induced maturation of DC, but other *B. pertussis* components stimulated DC maturation independently of TLR4. The course of *B. pertussis* infection was more severe in TLR4-defective, C3H/HeJ mice compared with C3H/HeN mice. Surprisingly, antibody and antigen-specific IFN-γ responses were enhanced at the peak of infection, whereas antigen-specific IL-10-producing T cells were significantly reduced in C3H/HeJ mice. This was associated with enhanced inflammatory cytokine production, cellular infiltration, and severe pathological changes in the lungs of TLR4-defective mice. These findings suggest that TLR4-mediated signalling activates innate IL-10 production in response to *B. pertussis* and promotes the induction of IL-10 secreting Tr1 cells. These Tr1 cells inhibit Th1 responses and limit inflammatory pathology in the lungs during infection with *B. pertussis*.

While the precise mechanism of protective immunity to *B. pertussis* induced by immunisation with Pw or Pa remains to be fully defined, it appears that Pa protects largely through antibody induction whereas Pw, which is more effective, induces both antibody and cell mediated immunity. TLR4-defective mice immunised with Pw failed to resolve infection within 14 days of challenge whereas C3H/HeN mice completely cleared the infection from their lungs within 10 days of challenge demonstrating an absolute requirement for TLR4 in mediating Pw-induced protection against *B. pertussis*. Although protection was observed in the TLR4-defective mice immunised with Pa, this protection was sub-optimal when compared with that seen in the C3H/HeN mice. DC from the TLR4-defective, C3H/HeJ mice had defective IL-1β, TNF-α and IL-12p40 in response to Pw and Pa *in vitro*. This suggests that activation of effector mechanisms of innate immune cells mediated through TLR4 is an essential component of vaccine-induced cellular immunity to *B. pertussis*.

Tracheal cytotoxin (TCT), a disaccharide-tetrapeptide monomer of peptidoglycan released by growing *B. pertussis*, induces cytopathology in hamster tracheal ring organ cultures similar to the pathology observed during pertussis disease. In this study, TCT was shown to interact with murine DC, and this interaction potentiated innate responses, especially IL-1β and IL-10 production in response to other TLR ligands *in vitro*. TCT was also shown to downregulate LPS-induced MHC class II expression on DC. Injection of TCT into mice suppressed the development of T cell responses induced by immunisation with a foreign antigen in the presence of alum or *Escherichia coli* heat labile enterotoxin as an adjuvant. Incubation of DC with TCT suppressed their ability to promote T cell responses following adoptive transfer *in vivo*. Together, these data indicate that the interaction of TCT with the DC may represent an immune evasion strategy by which *B. pertussis* attempts to subvert protective immune responses.

In conclusion, interaction of *B. pertussis* virulence factors with pathogen recognition receptors on innate cells is a critical component of protective immunity to *B. pertussis* but can also be exploited by the bacteria to subvert the immune responses of the host.

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ABBREVIATIONS

ACT	Adenylate Cyclase Toxin
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
AKP	Alkaline Phosphatase
APC	Antigen Presenting Cell
BAL	Broncheoalveolar Lavage
BCG	Bacille Calmelle-Guérin
BMDC	Bone marrow-derived DC
Bp SON	*B. pertussis* sonicate
BCW	Bacterial Cell Wall
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CARD	Caspase Activation and Recruitment Domain
CD	Cluster of Differentiation
CFU	Colony Forming Units
CpG	Cysteine-phosphate-Guanosine motif
cpm	counts per minute
CR3	Complement Receptor 3
CT	Cholera Toxin
CTLA-4	cytolytic T lymphocyte–associated antigen 4
CyaA	Adenylate Cyclase Toxin
DAP	diaminopilimic acid
DC	Dendritic Cell

DNA	Deoxyribonucleic Acid
DMSO	dimethyl sulphoxide
DT	Diphtheria Toxoid
DTP	Diphtheria, Tetanus, Pertussis
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalitis
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FHA	Filamentous Hemagglutinin
FITC	Fluorescein Isothiocyanate
FIM	Fimbrae
FSON	Formalin treated *B. pertussis* sonicate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
H&E	Haematoxytin and Eosin
HRP	Horseradish Peroxidase
i.c	intracerebral
i.n	intranasal
i.p	intraperitoneal
Ig	Immunoglobulin
IL-	Interleukin
IFN	Interferon
iNOS	inducible Nitric Oxide
KLH	Keyhole Limpet Haemocyanin

LPS	Lipopolysaccharide
LOS	Lipooligosaccharide
LT	Heat labile toxin of *Escherichia coli*
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatability Protein
MIP	Macrophage Inflammatory Protein
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor-κB
ng	nanogram
N IBSC	National Institute for Biological Standards and Controls
NO	Nitric Oxide
NOD	Nucleotide Binding Oligomerization Domain
NK	Natural Killer Cell
OD	Optical Density
OVA	Ovalalbumin
OPD	O-phenylendiamine
Pa	Acellular Pertussis Vaccine
PAMP	Pathogen-Associated Molecular Pattern
Pam-3Cys4	Palmitoyl-3-cysteine-serine-lysine-4
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pg	picogram
PGN	Peptidoglycan
PBMC	Peripheral Blood Mononuclear Cells
PMA	Phorbal Myristic Acid

PMN	Polymorphonuclear
PRN	Pertactin
PRR	Pathogen Recognition Receptor
PT	Pertussis Toxin
Pw	Whole Cell Pertussis Vaccine
RANTES	Regulation on Activation Normal T cell Activation
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s.c	sub-cutaneously
SD	Standard Deviation
SIDS	Sudden Infant Death Syndrome
STAT	Signal Transducer and Activation of Transcription
TB	Tuberculosis
TCR	T cell receptor
TCT	Tracheal Cytotoxin
TGF	Transforming Growth Factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
Tr1	T regulatory type 1
TT	Tetanus Toxoid
UV	Ultra Violet
WHO	World Health Organisation

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Chapter 1

General Introduction

I. GENERAL INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

Pathogen invasion presents the host with several challenges. Firstly, it must sense the pathogen and secondly, deliver an appropriate immune response. Protection against infection hinges on the close interplay between the innate and adaptive immune systems. Adaptive immunity is mediated by T- and B- lymphocytes that proliferate clonally in response to a specific pathogen or antigen. The generation of adaptive immune responses is advantageous to the host because of high affinity recognition and the generation of memory. However, a major disadvantage of adaptive immunity is that the response is gradual. Before the onset of adaptive immunity, the innate immune response takes centre stage. Innate immunity is a rapid response and therefore, represents the first line of defence to an invading pathogen or foreign antigen. The recent discovery of a class of receptors involved in the recognition of molecular structures characteristic of groups of microorganisms has highlighted the innate immune system as a discriminating system. In addition, the innate immune response has a decisive impact on the type of adaptive immune response (Fearon, 1997; Medzhitov *et al.*, 1997).

1.2 INNATE IMMUNE RECOGNITION

Innate immune recognition is based on the detection of conserved molecular structures produced by microorganisms but not by the host (Janeway, 1989). These structures were originally called pathogen-associated molecular patterns (PAMPs) and

the receptors that recognised these PAMPs were termed pattern-recognition receptors (PRRs) (Janeway, 1989; Medzhitov *et al.*, 1997). PAMPs may not however, be an appropriate term as they are also expressed by commensal microorganisms and specific molecules and not 'patterns' are actually recognised. The term 'microbial activator' instead of PAMP has been recently proposed (Beutler, 2003). The best known microbial molecular structures that activate innate immunity include lipopolysaccharide (LPS) of gram-negative bacteria, lipoteichoic acids (LTA) of gram-positive bacteria, lipoproteins, double stranded (ds) RNA, produced by many viruses during their infection cycle, and β-glucans and mannans found in the fungal cell wall. All of these structures are produced by different classes by microorganisms, but importantly, not by the host organism. Therefore, they function as 'molecular signatures' of microorganisms and their recognition by the innate immune system signals the presence of infection (Medzhitov and Janeway, 2002; Takeda and Akira, 2005).

1.2.1 RECEPTORS OF THE INNATE IMMUNE SYSTEM

The innate immune system detects infection using a variety of PRRs that recognise microbial molecular structures and trigger various effector responses (Medzhitov *et al.*, 1997). Several classes of PRRs have evolved to perform this function and may be categorised into three functional classes (Medzhitov, 2001).

i) PRRs that signal the presence of infection

These PRRs can be expressed on the cell surface or intracellularly. The best known receptors of this class are the toll-like receptor family (section 1.2.2) and the intracellular nucleotide-binding oligomerization domain (NOD) proteins.

ii) Phagocytic (or endocytic) PRRs

These PRRs are expressed on the surface of dendritic cells (DC), macrophages, and neutrophils. They recognise microbial molecular structures on the surface of pathogens and mediate their uptake. Examples include the macrophage mannose receptor (MR) and the macrophage receptor with collagenous structure (MARCO).

iii) Secreted PRRs

PRRs of this class perform three functions:

(a) they activate complement

(b) they opsonize microbial cells to facilitate phagocytosis

(c) they function as accessory proteins for microbial activator recognition by transmembrane receptors

Examples of these PRRs include the mannan-binding lectin (MBL) and the peptidoglycan recognition proteins (PGRPs).

1.2.2 TOLL-LIKE RECEPTORS (TLRS)

TLRs represent a crucial component of the innate immune response. Their discovery arose from the observation that activation of a receptor, toll, in the fruit fly, *Drosophila-melanogaster* lead to the induction of an anti-fungal agent, drosomycin (Lemaitre *et al.*, 1996). The first mammalian TLR was cloned in 1994 by Nomura and co-workers (Nomura *et al.*, 1994). In 1996, the gene encoding this protein, later known as TLR1, was mapped to chromosome 4 in humans (Taguchi *et al.*, 1996). To date, mammalian TLRs comprise a family of at least 11 members. TLR1-9 are conserved between the human and the mouse (Takeda and Akira, 2005).

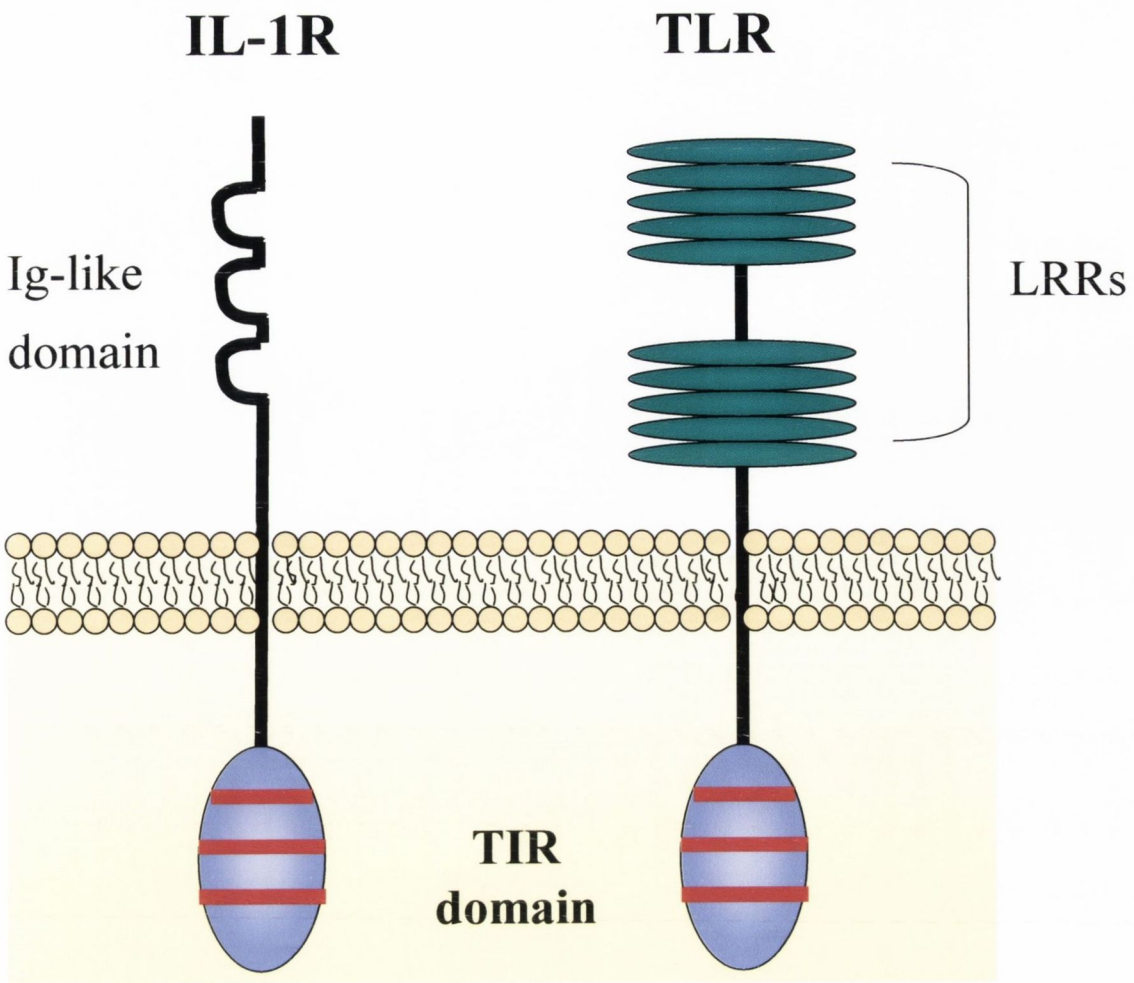


Figure 1.1 **TLR and IL-1R families share a homologous intracellular TIR domain.**

TLRs comprise a family of type I transmembrane receptors that are characterised by leucine rich repeats (LRRs) in the extracellular portion and an intracellular Toll/IL-1 receptor (TIR) domain, which is homologous to the intracellular domain of IL-1 receptor family members (Akira *et al.*, 2001; Medzhitov, 2001). Since their discovery, it has been established that TLRs play important roles in recognising specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses.

1.2.3 TLR4 AND THE LPS SIGNALLING COMPLEX

Studies attempting to elucidate the LPS signalling pathway have made a major contribution to our current understanding of the innate immune response. In 1965, it was observed that mice of the C3H/HeJ strain were highly resistant to the biological effects of LPS (Heppner G and DW., 1965). Detailed genetic studies revealed that C3H/HeJ mice had a single co-dominant mutation that interfered with the LPS response. This mutation was mapped to chromosome 4 in 1978 (Watson *et al.*, 1978a; Watson *et al.*, 1978b). A mutation occurring in the C57BL/10ScCr strain of mice was also shown to affect LPS sensitivity. This mutation was shown to occur in the same locus as the C3H/HeJ mutation. This locus was termed Lps and the mutant allele that impaired the LPS responses was termed Lps^d (Coutinho A and T., 1978).

Another key discovery in the LPS field was the observation that the lethal effects of LPS were entirely conferred by cells of the haematopoietic origin (Michalek *et al.*, 1980). LPS binding protein (LBP) was identified as a plasma protein that binds to the lipid A moiety of LPS (Tobias *et al.*, 1986). The generation of LBP-deficient mice revealed a non-redundant role for LBP in the response to LPS (Jack *et al.*, 1997;

Wurfel *et al.*, 1997). The formation of an LPS and LBP complex was shown to trigger the association of this complex with another LPS-binding molecule, CD14 (Tobias and Ulevitch, 1993).

The importance of CD14 in the response to LPS has been demonstrated in CD14-deficient mice, these mice exhibited a reduced response to LPS (Haziot *et al.*, 1996; Moore *et al.*, 2000). CD14 has no cytoplasmic domain and therefore can not transduce an LPS signal from the membrane to the cytoplasm. The first discovered mammalian homologue of *Drosophila* Toll was found to work downstream from CD14 and is principally responsible for delivering an LPS signal (Medzhitov *et al.*, 1997). Poltorak and co-workers identified the mutation that was responsible for the LPS-hyporesponsive trait in C3H/HeJ mice. In C3H/HeJ mice, a point mutation modifies a conserved residue of the Tlr4 cytoplasmic domain (P712H) and C57BL/10ScCr mice lack the entire genomic region for the Tlr4 gene (Poltorak *et al.*, 1998b; Poltorak *et al.*, 1998a).

Although TLR4 is indispensable for LPS signalling, a requirement for another molecule was suggested by *in vitro* studies. *In vitro* transfection of TLR4 cDNA did not confer LPS responsiveness to LPS-unresponsive cell lines, human embryonic kidney-derived 293 and a mouse IL-3 dependent pro-B cell line Ba/F3 (Kirschning *et al.*, 1998) (Shimazu *et al.*, 1999). This was subsequently explained by the absence of another component of the LPS recognition complex, MD-2 (Shimazu *et al.*, 1999) . Recently, the TLR4 region Glu24 – Pro34 was identified as critical for interaction with MD-2 (Nishitani *et al.*, 2005) . Together, the TLR4/MD-2/CD14-LBP complex forms the LPS signalling apparatus.

1.2.4 TLR4 - MORE THAN AN LPS SENSOR

In addition to LPS recognition, TLR4 is implicated in the recognition of taxol, a diterpene purified from the bark of the western yew, *Taxus brevifolia* (Byrd-Leifer *et al.*, 2001; Kawasaki *et al.*, 2000). Furthermore, TLR4 can sense a protein (fusion protein) from the respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000). TLR4-deficient mice cannot eradicate RSV efficiently suggesting that TLR4 is also plays a protective role against viral infection. Vaccinia virus carries two protein products (A46R and A52R) with similar amino acid structures to TIR domains, through which IL-1 and TLR4 signalling can be inhibited (Bowie *et al.*, 2000).

TLR4 has also been shown to be involved in the recognition of host-derived products. During inflammation or tissue injury, extracellular matrix components, such as fibronectin or collagen are degraded by proteases. These degraded products then trigger inflammatory responses. One of these products, a fragment of fibrinectin has been shown to signal through TLR4 (Okamura *et al.*, 2001). Fibrinogen has also been shown to stimulate chemokine production from macrophages in a TLR4-dependent manner (Smiley *et al.*, 2001). TLR4 therefore appears to sense several proteins generated during the inflammatory response.

Endogenous molecules such as heat shock proteins (HSPs) are released upon necrotic but not apoptotic cell death (Basu *et al.*, 2000). TLR4 has been shown to be involved in sensing HSP60 (Ohashi *et al.*, 2000; Vabulas *et al.*, 2001). The production of inflammatory cytokines by HSP70 was impaired in TLR4-deficient mice (Asea *et al.*, 2002). However, it has been reported that contamination of HSP70 preparation with LPS conferred the ability to activate TLR4 (Gao and Tsan, 2003a).

Interestingly, if it is confirmed that endogenous host molecules, free from any other microbial contaminants, can act as TLR ligands, this would provide a molecular basis for the 'danger' theory proposed by Matzinger (Matzinger, 1994). Matzinger argues that the innate immune system can sense danger signals that are mediated by certain classes of stimulatory tissue factors and the response of these innate cells, in turn induces adaptive immunity.

1.2.5 Co-operative TLRs: TLR1, TLR2 and TLR6

TLR2 recognises a wide range of microbial molecules. TLR2 has been implicated in the recognition of bacterial lipoproteins (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999), a phenol soluble modulin from *Staphylococcus epidermis* (Hajjar *et al.*, 2001), zymosan from yeast cell walls (Underhill *et al.*, 1999), glycosylphosphotidylinositol (GPI)-anchors from the membrane of *Trypanosoma cruzi* (Campos *et al.*, 2001). In addition, TLR2 has been reported to recognise LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* and *Helicobacter pylori* (Hirschfeld *et al.*, 2001; Smith *et al.*, 2003; Werts *et al.*, 2001). LPS from these bacteria differ from LPS from other bacteria in the number of acyl chains in their lipid A moiety, which is thought to explain this differential recognition (Netea *et al.*, 2002). A recent study has however, reported that the contaminating lipoproteins in the LPS preparation from *P. gingivalis* activate TLR2 and that highly pure *P. gingivalis* LPS is a weak activator of TLR4 (Hashimoto *et al.*, 2004).

TLR2 is known to form heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2 (Ozinsky *et al.*, 2000). TLR1 and TLR2 association has been shown to recognise triacyl lipoproteins,

whereas TLR2 and TLR6 co-operate in the recognition of diacyl lipoproteins (Takeuchi *et al.*, 2001).

1.2.6 TLR3

TLR3 has unique structural features among the TLRs discovered to date. TLR3 lacks the proline residue that is conserved among the other TLRs. This is the proline residue where the mutation occurs in the Tlr4 gene of the LPS-hyporesponsive C3H/HeJ mice (Takeda *et al.*, 2003). TLR3 is also unique in that it is preferentially expressed on mature myeloid DC (section 1.5) (Muzio *et al.*, 2000). TLR3-deficient mice were shown to exhibit an impaired response to dsRNA and synthetic dsRNA (poly I:C) (Alexopoulou *et al.*, 2001). DsRNA is associated with viral infections, as most viruses produce dsRNA at some point in their infection cycle. TLR3 therefore appears to have a role in alerting the host to viral infection.

1.2.7 TLR5

TLR5 recognises flagellin, the protein subunit that make up bacterial flagella, which aid bacterial mobility (Hayashi *et al.*, 2001). Increased susceptibility to pneumonia caused by the flagellated bacterium *Legionella pneumophilia* has been linked to a common stop codon polymorphism in the ligand binding domain of TLR5 (Hawn *et al.*, 2003).

1.2.8 TLR7 AND TLR8

Several synthetic imidazoquinolines have been demonstrated to have potent antiviral and antitumour effects due to their ability to induce inflammatory cytokines, especially IFN-α. One of these imidazoquinoline compounds, Imiquimod, has been approved for the treatment of human papillomavirus infection (Hemmi *et al.*, 2002). Hemmi and co-workers reported that TLR7-deficient mice do not respond to synthetic imidazoquinolines (Imiquimod and R848) (Hemmi *et al.*, 2002). Human but not murine, TLR8 has been shown also to recognise imidazoquinoline (Jurk *et al.*, 2002). Another synthetic compound, loxoribine is also recognised by murine TLR7 (Heil *et al.*, 2003; Lee *et al.*, 2003). Imidazoquinoline and Ioxoribine are both structurally related to guanosine nucleoside. Recently, it was reported that TLR7 and TLR8 recognise uridine-rich single-stranded (ss) RNA from viruses such as human immunodeficiency virus and influenza virus (Heil *et al.*, 2004; Lund *et al.*, 2004).

1.2.9 TLR9

Prokaryotic DNA contains unmethylated CpG motifs, which have immunostimulatory activity. In eukaryotes, the frequency of CpG motifs is severely reduced and the cysteine residues of the CpG motifs are highly methylated (Wagner, 1999). Using TLR9-deficient mice, TLR9 was identified as the signal transducing receptor for CpG (Hemmi *et al.*,2000). In addition to bacterial DNA, TLR9 has been shown to also recognise viral CpG on plasmacytoid DC (section 1.5) (Krug *et al.*, 2004; Lund *et al.*, 2003). Furthermore, TLR9-deficient mice have been shown to be susceptible to mouse cytomegalovirus (MCMV) infection (Tabeta *et al.*, 2004).

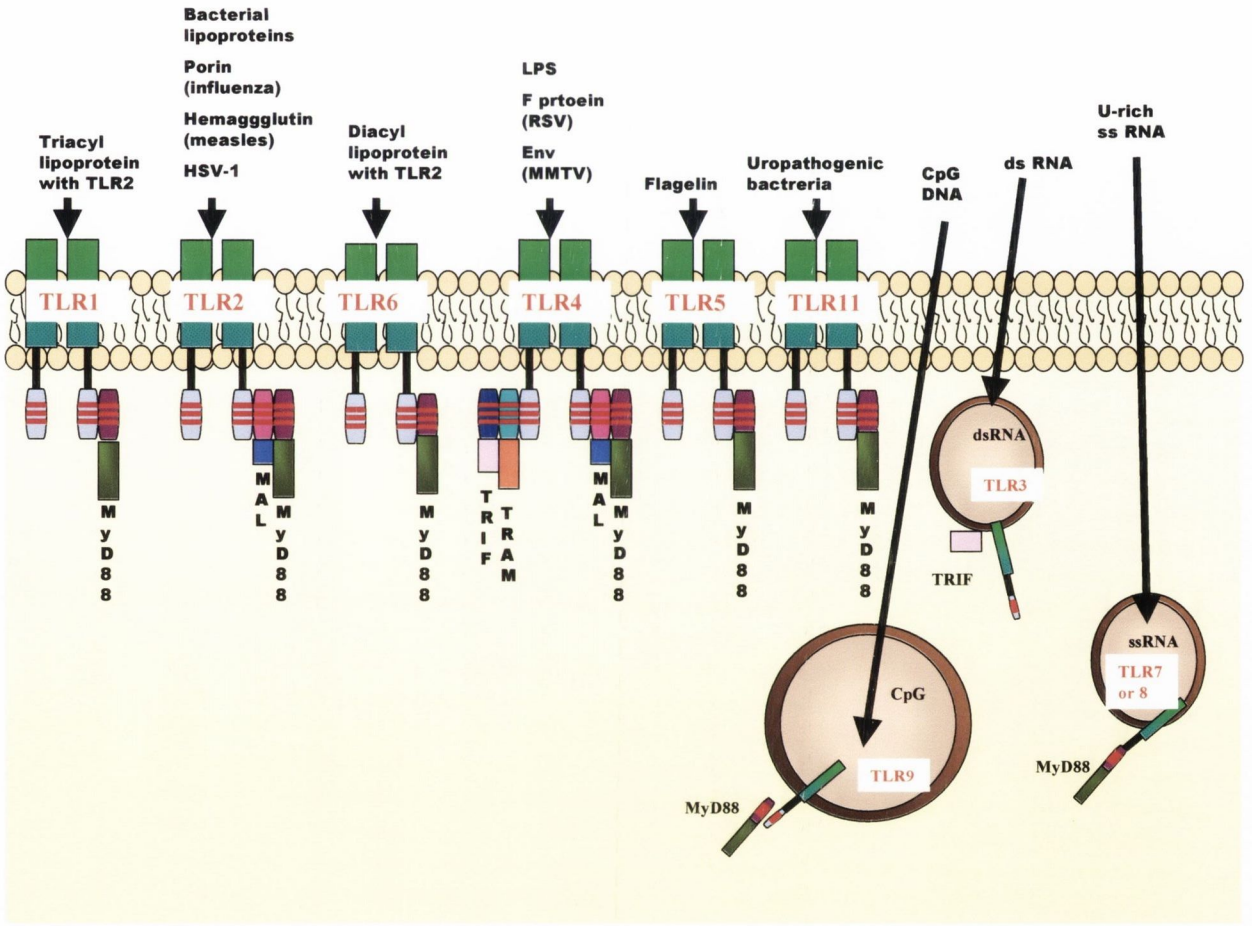


Figure 1.2 Members of the TLR family and their ligands.

1.2.10 TLR11

TLR11-deficient mice are highly susceptible to uropathogenic bacterial infection (Zhang *et al.*, 2004). Although no specific ligand has been identified for TLR11, these findings implicate TLR11 in mediating an anti-uropathogenic bacterial response.

1.3 TLR SIGNALLING PATHWAYS - AN OVERVIEW

Activation of TLRs by microbial components leads to the induction of numerous genes that function in inflammatory and immune responses. These include inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-12), chemokines (MIP-1α, MIP-2), up-regulation of MHC and co-stimulatory molecule expression on the cell surface (Akira *et al.*, 2001; Medzhitov, 2001; Medzhitov *et al.*, 1997).

Both the TLR and IL-1R families activate similar signalling pathways as they both contain TIR domains in their intracytoplasmic regions. One of the first steps in the signalling process involves the recruitment of TIR domain containing adaptor molecules to the TIR domain of the TLR.

1.3.1 MyD88 DEPENDENT PATHWAYS

Myeloid differentiation factor 88 (MyD88) can interact with the TIR domain of all TLR family members with the exception of TLR3. The TIR domain of MyD88 is located in its C-terminal portion, but has a death domain in its N-terminal instead of the transmembrane domain found in TLRs (Muzio *et al.*, 1997; Takeda *et al.*, 2003).

Upon stimulation, MyD88 recruits a death-domain containing serine/threonine kinase, the IL-1R-associated kinase (IRAK)-1. In addition to IRAK-1, two additional kinases, IRAK-2 and IRAK-4 have been reported to function in the TLR/IL-1R signalling pathways (Muzio *et al.*, 1997; Wesche *et al.*, 1999). The existence of IRAK-2 and -4 may account for the fact that mice deficient in IRAK1 have only a mild defect in TLR signalling (Thomas *et al.*, 1999). Activation of IRAK1 by phosphorylation facilitates the interaction and activation of tumour necrosis factor receptor-associated factor 6 (TRAF6) (Cao *et al.*, 1996). TRAF-6 in turn associates and activates TGF-β-activated kinase 1 (TAK1). TRAF-6-activated TAK1 phosphorylates IKKβ, which leads to the phosphorylation of the NF-κB pathway (Wang *et al.*, 2001). The NF-κB family of transcription factors play a critical role in innate immunity (Silverman and Maniatis, 2001). In addition, TRAF6 activated TAK1 may also phosphorylate the mitogen activated protein (MAP) kinase kinase 6 (MKK6), which in turn phosphorylates the MAP kinase, JNK leading to the activation of the API pathway (Wang *et al.*, 2001).

1.3.2 THE MYD88 INDEPENDENT PATHWAY

The existence of an MyD88-independent pathway was suggested by the observations that macrophages from MyD88-deficient mice failed to produce any inflammatory cytokines (e.g. TNF-α, IL-6) in response to LPS, but did retain the ability to activate NF-κB and MAP kinases, albeit with delayed kinetics (Kawai *et al.*, 1999). MyD88-deficient DC showed some characteristics of maturation i.e. enhanced expression of co-stimulatory molecules such as CD40 and CD86 in response to LPS (Kaisho and Akira, 2001; Kaisho *et al.*, 2001). These results indicate that cytokine

production induced by TLR signalling is dependent on an MyD88-mediated signalling pathway and that TLR4 can induce DC maturation in an MyD88-independent manner. Furthermore, since LPS-dependent activation of IFN regulatory factor (IRF)-3 is preserved in MyD88-deficient cells, IRF-3 activation may contribute to the MyD88-independent pathway (Kaisho *et al.*, 2001). Other adaptor molecules that participate in the MyD88-independent pathway are discussed in section 1.3.3.

1.3.3 ADAPTOR PROTEINS RECRUITED IN TLR4-MEDIATED SIGNALLING

To date, four TIR domain containing adaptor proteins are known to mediate TLR4 signalling. MyD88-adaptor-like (Mal) protein, also called TIR domain containing adaptor protein (TIRAP) was discovered based on its ability to mediate TLR4 but not IL-1R signalling (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). Cytokine production in LPS stimulated Mal-deficient macrophages is severely impaired (Horng *et al.*, 2002; Yamamoto *et al.*, 2002). TLR2 signalling in MAL-deficient macrophages was also impaired (Horng *et al.*, 2002; Yamamoto *et al.*, 2002). MAL is not required for TLR5, TLR7. or TLR9 signalling (Horng *et al.*, 2002). It is unclear if in this case MyD88 is acting alone or paired to a second, as yet unidentified receptor. TIR-domain containing adaptor inducing interferon-β (TRIF), also known as TIR domain containing adaptor molecule 1 (TICAM-1) has been identified as a key adaptor protein in the MyD88-independent pathway (Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2003). TRIF has also been shown to link TLR3 and TLR4 to the kinase complex IKKε/TBK1 which in turn activates the transcription factor, IRF3 (Fitzgerald *et al.*, 2003a; Sharma *et al.*, 2003). IRF3 is responsible for the induction of a group of genes specific for TLR3 or TLR4, such as IFNβ, or Interferon producing protein 10

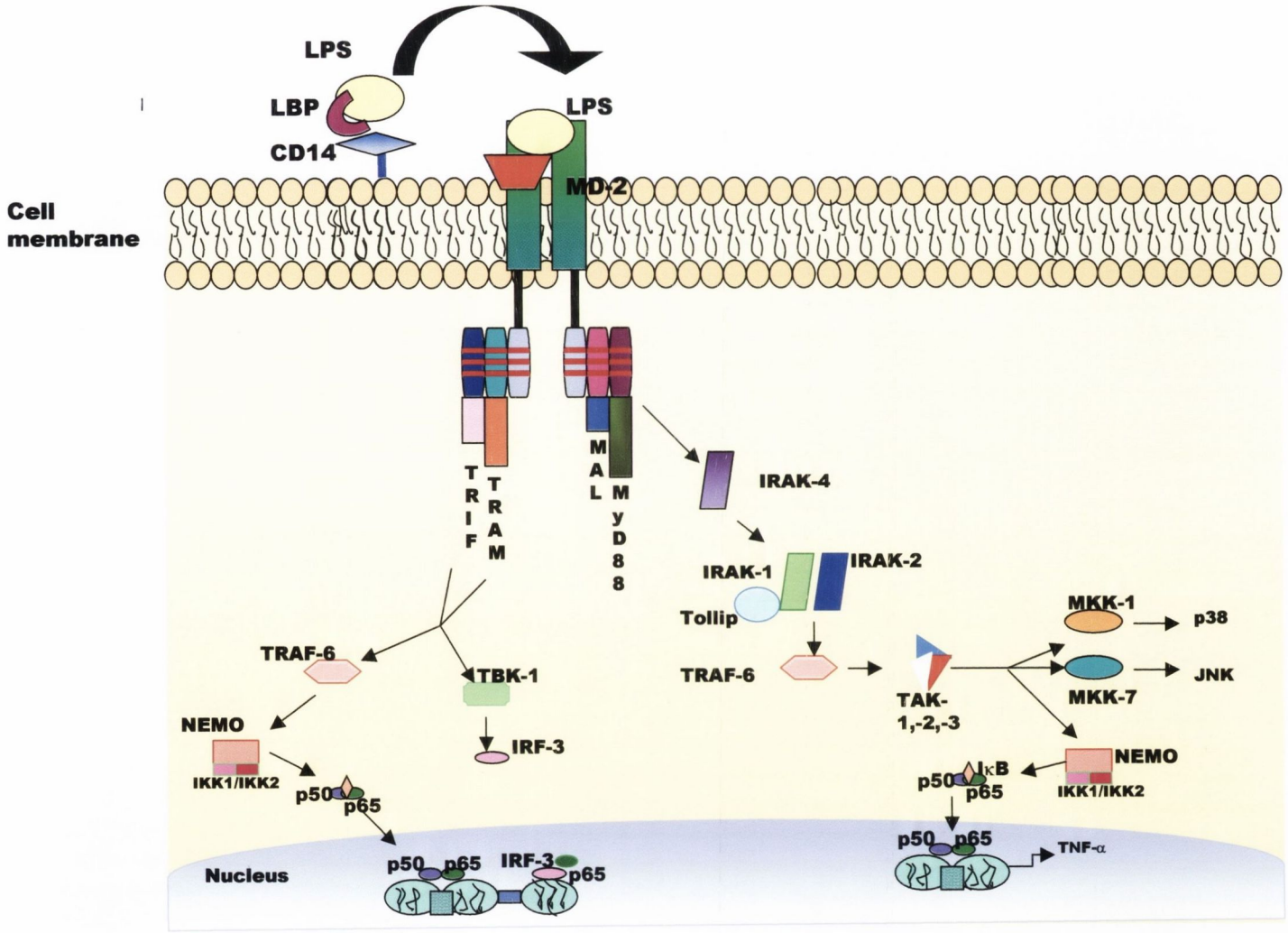


Figure 1.3 Activation of NF-κB through LPS-induced TLR4 mediated signalling.

(IP-10) (Doyle *et al.*, 2002; Kawai *et al.*, 2001). The induction of inflammatory cytokines by LPS (MyD88 dependent response), is also abolished in TRIF-deficient macrophages, suggesting that TRIF plays a role in both the MyD88-dependent and -independent pathways. Another adaptor molecule, TRIF-related adaptor molecule (TRAM) also known as TIR containing protein (TIRP) or TIR domain containing adaptor molecule (TICAM) was recently identified (Bin *et al.*, 2003; Fitzgerald *et al.*, 2003b; Oshiumi *et al.*, 2003). Fitzgerald and co-workers (2003) demonstrated using an siRNA technique that MyD88-independent signalling from the TLR4 receptor was regulated by both TRIF and TRAM (Fitzgerald *et al.*, 2003b). To date, MyD88, MAL, TRIF and TRAM all have a role to play in transducing an integrated signal in response to LPS.

1.4 INTRACELLUALAR SENSORS OF INFECTION

Recently, the nucleotide-binding oligomerization domain (NOD) proteins of proteins have been implicated in intracellular recognition of bacterial components. The NOD family of intracellular proteins are characterised by a nucleotide-binding domain followed by a leucine rich repeat region (Bertin *et al.*, 1999; Inohara and Nunez, 2001b; Ogura *et al.*, 2001a). Philpott *et al.* (2001) have proposed that NODs play a key role in host defence in tissues where TLRs are absent or expressed at low levels (Philpott *et al.*, 2001). For example, on cells such as colonic epithelial cells, which are in constant contact with microbial flora, expression of TLRs is down regulated in order to avoid stimulation of cells by commensal microorganisms and thereby avoid unnecessary inflammatory responses. However, where these cells to be infected with an invasive pathogen such as *Shigella flexneri*, NOD proteins recognise

the threat and mount an immune response (Girardin *et al.*, 2001). The best characterised members of this family are NOD1 and NOD2.

NOD1 contains only one CARD domain whereas NOD2 has an additional CARD domain (Ogura *et al.*, 2001a). The expression of NOD1 is ubiquitious, whereas NOD2 expression is restricted to monocytes/macrophages and epithelial cells (Bertin *et al.*, 1999; Inohara *et al.*, 1999). Recent studies have identified that NOD1 recognises purified peptidoglycan from gram-negative bacteria such as *Escherichia coli* and *Shigella flexneri* (Girardin *et al.*, 2003a). The peptidoglycan fragment recognised by NOD1 is a disaccharide of N-acetyl glucosamine-N-acetyl muramic acid linked to a tripeptide where the terminal amino acid is meso-diaminopimelic acid (meso-DAP) (Girardin *et al.*, 2003a). The presence of DAP in the peptidoglycan may be considered as a signature of gram-negative bacterial infection as most (but not all) bacteria have a lysine in this position in their peptidoglycan (Girardin *et al.*, 2003a; Girardin *et al.*, 2003b). NOD1 is highly specific for the tripeptide structure since the presence of an additional amino acid to the DAP abrogates the sensing of this bacterial product by NOD1 (Girardin *et al.*, 2003b). Recently, the minimal motif recognised by NOD1 was further defined. NOD1 detects the terminal two amino acids within the d-glutamine-meso-DAP (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003c).

NOD2 is regarded as a general sensor for both gram-positive and gram-negative bacteria, since it recognises muramyl dipeptide (MDP) - a motif found in all peptidoglycans (Girardin *et al.*, 2003b; Inohara and Nunez, 2003). In addition to MDP, NOD2 can also detect muramyl-triLys but not muramyl-triDAP (Girardin *et al.*, 2003c). Recently, it was demonstrated that a mutation in NOD2 is associated with Crohns disease, a common inflammatory disease of the intestinal tract (Hampe *et al.*,

2002; Hugot *et al.*, 2001). Patients with this mutation have a reduced ability to produce the anti-inflammatory cytokines, IL-10 and TGF-β (Netea *et al.*, 2004). Furthermore, a NOD2 mutation has also been found to potentiate NF-κB activation and IL-1β processing (Maeda *et al.*, 2005).

1.4 DENDRITIC CELLS AS PIVOTAL CELLS IN THE IMMUNE RESPONSE

DC play a key role in the initiation and the regulation of T-cell dependent immune responses. Much of their significance lies in their role as a cell that links the evolutionary ancient innate immune system to the adaptive immune system. DC are derived from haematopoietic stem cells in the bone marrow and form a network of heterogeneous cell populations. DC can be separated into subsets, which differ in phenotype, function and location (Shortman and Liu, 2002). In humans, myeloid DC include epidermal langerhan cells, and plasmacytoid DC have been described. Murine DC express the CD11c integrin and can be subdivided on the basis of CD8α expression. A population of murine $CD11c^+$ cells also expressing B220 and Gr-1, are equivalent to human plasmacytoid DC (Colonna *et al.*, 2002), whereas murine myeloid DC are $CD11c^+$, $CD8\alpha^-$, $CD11b^+$ (Pulendran, 2004). $CD8\alpha^-$, $CD8\alpha^+$, along with plasmacytoid $B220^+$ DC share a common origin in that they can be generated by both myeloid and lymphoid precursors (Ardavin, 2003; Traver *et al.*, 2000). The precursors migrate to various tissues, where they differentiate into immature DC (Banchereau *et al.*, 2000a; Kalinski *et al.*, 1999; Moser and Murphy, 2000; Pulendran *et al.*, 2001a). Immature DC are viewed as the sentinels of the immune system. The ability of DC to act as immunological sensors is achieved through the expression of PRRs (Moser and Murphy, 2000; Pulendran *et al.*, 2001a).

At the immature stage, DC exhibit potent endocytic activity. DC switch from their specialised antigen-capturing function to T cell activation mode in a process termed maturation. DC migrate from the tissue to the lymph nodes and undergo profound changes during maturation. The endocytic capacity of the DC is downregulated, while there is a marked upregulation of MHC and costimulatory molecule expression (Moll, 2004). In addition, mature DC express enhanced surface expression of CD80, CD86 and CD40 (Banchereau *et al.*, 2000b). The expression of inflammatory chemokine receptors, such as CCR1, CCR2, CCR5 and CCR6 decreases. Maturing DC enhance their cell surface expression of CCR7 and thereby their responsiveness to its ligands, CCL19 (MIP-3β) and CCL21 (secondary lymphoid tissue chemokine, SLC) that are produced in the T cell areas of the lymph nodes (Dieu *et al.*, 1998; Saeki *et al.*, 1999; Vecchi *et al.*, 1999). As CCR7 also mediates homing of T cells to secondary lymphoid organs, it is a key receptor for the encounter of antigen bearing mature DC and responder T cells (Willimann *et al.*, 1998).

1.5.1 TLR EXPRESSION ON DC SUBPOPULATIONS

TLRs are differentially expressed on human and murine DC subsets. In mice, all DC subsets isolated from the spleen express TLR -1, -2, -4, -6, -8, and -9 (Edwards *et al.*, 2003). Murine plasmacytoid $B220^+$ DC do not express TLR3. Murine mouse $CD8\alpha^+$ DC lack TLR5 and TLR7 expression (Doxsee *et al.*, 2003; Edwards *et al.*, 2003).

In humans, plasmacytoid DC express TLR7 and TLR9, whereas myeloid DC express TLR-1, -2, -3, -5, -6 and -8 (Hornung *et al.*, 2002; Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001). In certain studies, TLR7 expression was expressed on both

plasmacytoid and myeloid DC (Ito *et al.*, 2002; Krug *et al.*, 2001), whereas others have reported that TLR7 expression is exclusively found on plasmacytoid DC (Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001).

DC LINK THE INNATE AND ADAPTIVE IMMUNE RESPONSES

DC play a central role in shaping the nature of the adaptive immune response. Recent studies have indicated that several factors can influence the development of polarized immune responses, including, DC lineage, activation status, and the DC-T cell ratio (Kalinski *et al.*, 1999; Maldonado-Lopez and Moser, 2001; Moser and Murphy, 2000; Pulendran *et al.*, 1999). Although it has been proposed that $CD8\alpha^+$ DC may be specialised for promoting Th1 responses and that CD8α- DC for Th2 responses (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999), subsequent studies have shown that distinct DC subsets are able to promote either type of T cell response, depending upon the pathogen-derived signals and host-derived cytokines present in the milieu (De Smedt *et al.*, 2001; MacDonald *et al.*, 2001; Maldonado-Lopez and Moser, 2001; Vieira *et al.*, 2000).

The fate of naïve T helper (Th) cells is determined by three signals that are provided by the pathogen-primed DC. Signal 1 is provided through the ligation of T cell receptors (TCRs) by MHC Class II molecules with bound peptide antigen on the DC surface. The initiation of protective immunity also requires T cell co-stimulation (Cunningham and Lafferty, 1977) involving interactions between CD28, CTLA4, ICOS and members of the B7 family, such as CD80, CD86 (Kroczek *et al.*, 2004). In the absence of co-stimulatory signal 2, Th cells become anergic. DC-derived cytokines represent the third signal and can act to bias the development of Th

response i.e. to a Th2, Th1 or T regulatory (Tr) phenotype (section 1.8). Th1-polarising cytokines include interleukin (IL) 12 family members; IL-12, IL-23 and IL-27 (Langrish *et al.*, 2004; Trinchieri, 2003) and type I interferons (IFNs) (Kadowaki *et al.*, 2000). Th2 polarising DC-derived factors include production of monocytic chemotactic protein 1 (MCP1; also known as CCL2) (Ohshima and Delespesse, 1997). Although, it was reported that DC-derived IL-4 may be required for Th2 priming (d'Ostiani *et al.*, 2000), this was later refuted by Maldonado-Lopez et al. (Maldonado-Lopez *et al.*, 2001). Adoptive transfer of DC from IL-4 deficient mice induce an unaffected Th2-type response (Maldonado-Lopez *et al.*, 2001). Co-stimulatory molecule expression of CD86 and OX40 ligand (OX40L) by DC have also been implicated in the development of a Th2 response (DeBecker *et al.*, 1998; Freeman *et al.*, 1995; Ranger *et al.*, 1996). DC-derived T regulatory polarising factors have been identified as IL-10 and transforming-growth factor (TGF-β) (McGuirk *et al.*, 2002; Zeller *et al.*, 1999). Therefore, through the upregulation of MHC and co-stimulatory molecules and the production of key cytokines and chemokines, DC play a pivotal role in orchestrating both the innate and adaptive responses to different pathogens.

1.5.2 TH POLARIZING CYTOKINE PRODUCTION BY DC

The cytokine milieu that is established during the DC mediated innate response to pathogens sets the stage for the adaptive immune response. Key cytokines released by the pathogen primed DC are instrumental in shaping the antigen-specific T cell response.

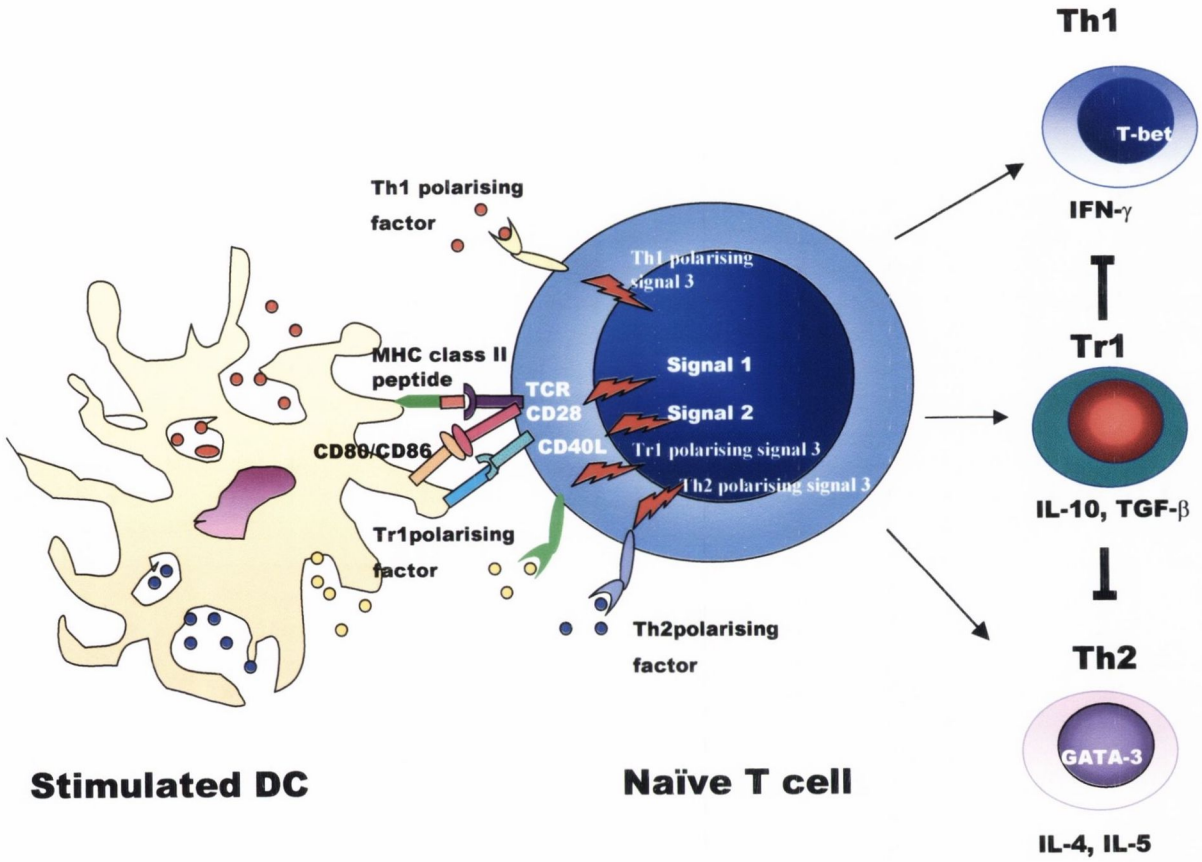


Figure 1.4 DC link the innate and adaptive immune responses

1.5.3.1 IL-12

Discovered in 1989, IL-12 is a heterodimeric cytokine of 70 kDa comprised of covalently linked p40 and p35 subunits (Kobayashi *et al.*, 1989). TLR agonists such as LPS, LTA and CpG are potent inducers of IL-12 production from DC and macrophages (Hochrein *et al.*, 2001; Pulendran *et al.*, 2001b; Shah *et al.*, 2003). In addition, IL-12 is induced by the engagement of CD40 on APCs with CD154 (CD40L) on activated T cells (Trinchieri, 2003). IL-12 has pleiotropic functions that promote cellular responses through a variety of mechanisms (Farrar *et al.*, 2002; O'Shea *et al.*, 2002; O'Shea and Paul, 2002). IL-12 induces the proliferation of T cells to a Th1 phenotype. IL-12 induces IFN-γ production by natural killer (NK) and T cells, an effect that is synergistically enhanced by the unrelated cytokine IL-18. The synergy between IL-12 and IL-18 is at least partially explained by the finding that each cytokine induces the receptor of the other (Lawless *et al.*, 2000; Smeltz *et al.*, 2001; Yoshimoto *et al.*, 1998). IL-18 was originally described as an IFN-γ releasing factor (Dinarello, 2000). In addition, IL-12 alone or in combination with IL-18, acts on APCs to induce IFN-γ and more IL-12 (Frucht *et al.*, 2001; Fukao *et al.*, 2001; Grohmann *et al.*, 1998; Grohmann *et al.*, 2001). This acts to further promote Th1 differentiation and cell-mediated responses.

The predominant source of IL-12 includes activated DC, monocytes and macrophages (Hsieh *et al.*, 1993; Macatonia *et al.*, 1995; Trinchieri *et al.*, 1992). Splenic DC have been shown to secrete IL-12p70 and stimulate Th1 cells (Macatonia *et al.*, 1993). However, within the DC subsets found in the spleen, the $CD8^+$ subset was shown to secrete much higher concentrations of IL-12p70 than $CD8^-$ DC *in vitro* (Pulendran *et al.*, 1997).

The IL-12 receptor (IL-12R) consists of two receptor units, β1 and β2 (Chua *et al.*, 1995; Presky *et al.*, 1996). Resting T cells do not express β1 or β2, but these are induced upon T cell activation. Mice deficient in either the IL-12 components (p35 or p40) or in its receptor components (IL-12R -β1 or -β2) have deficient Th1 responses, confirming IL-12 as a critical Th1 polarising cytokine *in vivo* (Trinchieri, 2003; Wu *et al.*, 1997; Wu *et al.*, 1996). However, studies that used depleting antibodies specific for IL-12p40 or mice that were deficient in IL-12p40 may have over emphasised the importance of IL-12 given the recent finding that the p40 homodimer can associate with a p19 subunit to form IL-23 (Oppmann *et al.*, 2000).

1.5.3.2 IL-23

The recently identified cytokine IL-23 has functions similar to, yet distinct from, those of IL-12 (Belladonna *et al.*, 2002; Oppmann *et al.*, 2000). IL-23 shares certain actions with IL-12, such as the induction of IFN-γ production in T cells (Belladonna *et al.*, 2002). Among the unique functions of IL-23 is the preferential induction of Th1 cells the memory subset of T cells (Belladonna *et al.*, 2002).

In contrast to IL-12, IL-23 does not use IL-12Rβ2 as part of its receptor (Parham *et al.*, 2002). IL-23 binds a receptor comprising of IL-12Rβ1 and a second subunit designated IL-23R, which shares homology to IL-12Rβ2. IL-23R is expressed at low levels on DC, monocytes, NK and T cells (Parham *et al.*, 2002).

1.5.3.3 IL-27

IL-27 is another heterodimeric cytokine from the IL-12 family (Pflanz *et al.*, 2002). IL-27 is composed of Epstein Barr virus-induced gene 3 (EB13) and the novel subunit p28. The p28 subunit is homologous to the p35 subunit of IL-12, whereas EBI3 is related to IL-12p40 and resembles a soluble cytokine receptor (Devergne *et al.*, 1997; Devergne *et al.*, 1998). IL-27 is produced by myeloid DC, with the highest concentrations produced by LPS activated monocytes and monocyte-derived DC. IL-27 preferentially induces proliferation of naïve not memory T cells (Pflanz *et al.*, 2002). IL-27 acts alone or in synergy with IL-17 to promote IFN-γ production and Th1 differentiation.

1.5.3.4 IL-10

IL-10 is a pluripotent cytokine with potent effects on numerous cell populations. IL-10 was first described in 1989 as a cytokine synthesis inhibiting factor (CSIF) (Fiorentino *et al.*, 1989). Although IL-10 exerts potent anti-inflammatory effects, more recent data has shown IL-10 to have stimulatory effects on certain populations. Due to this 'dual role' of IL-10, this cytokine is more accurately described as an immunoregulatory cytokine.

APCs and lymphocytes are the primary targets of IL-10. IL-10 promotes the development of a Th2 cytokine pattern by inhibiting the IFN-γ production by T lymphocytes, particularly via the suppression of IL-12 (Moore *et al.*, 2001). IL-10 suppresses pro-inflammatory cytokine production by DC, and monocytes/macrophages (de Waal Malefyt *et al.*, 1991; Fiorentino *et al.*, 1991;

Romagnani, 1995). The presence of IL-10 during activation of $CD4^+$ T cells results in the development of a Tr cell (section 1.8) (Groux *et al.*, 1997; Levings *et al.*, 2001a; Levings *et al.*, 2001b; Zeller *et al.*, 1999).

It was suggested that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting the transcription factor, NF-κB. IL-10 was reported to block nuclear translocation of the NF-κB p65/p50 heterdimer in monocytes/macrophages (Clarke *et al.*, 1998; Wang *et al.*, 1995). Recently, it was reported that IL-10 exerts its anti-inflammatory effect in part, by a selective induction of p50 nuclear translocation while blocking translocation of the p65/p50 heterodimer (Driessler *et al.*, 2004).

The effect of IL-10 on NK cells is mainly stimulatory. IL-10 favours the cytotoxic activity of these cells. IL-10 increases production of cytokines such as IFN-γ and TNF-α from NK cells (Carson *et al.*, 1995). IL-10 has various but weak effects on B cells. It prevents apoptosis and enhances the proliferation and differentiation toward plasma cells as well as IgM synthesis (Levy and Brouet, 1994; Rousset *et al.*, 1995). IL-10 has been shown to also have a role in immunoglobulin (Ig) class switching. In combination with IL-4, IL-10 induces IgG4 but inhibits IgE production and in combination with TGF-β, IL-10 induces IgA1 and IgA2 secretion (Defrance *et al.*, 1992; Jeannin *et al.*, 1998).

IL-10 deficient mice develop lethal inflammation of the intestine, which can be prevented by IL-10 administration (Kuhn *et al.*, 1993). Studies have shown that IL-10 can suppress Th1-mediated inflammatory conditions, such as colitis. However, IL-10 administration was only successful when administered before initiation of colitis, but was ineffective at reversing any established inflammation (Barbara *et al.*, 2000; Herfarth *et al.*, 1996; Powrie *et al.*, 1993). IL-10 treatment has also been shown

to be beneficial in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis (Chen *et al.*, 1994; Rott *et al.*, 1994) .

IL-10 can also exert protective effects during infection, by preventing uncontrolled inflammatory responses to certain pathogen-derived molecules. IL-10 deficient mice exhibit a prolonged inflammatory response to acute *Pseudomonas* challenge resulting in neutrophil accumulation in the lung (Chmiel *et al.*, 2002).

1.5.3.5 IL-6

IL-6 is produced by a number of activated cell types including DC, macrophages, Th2 and B cells. IL-6 binds to the surface receptor IL-6Rα, leading to the dimerisation of gp130/IL-6Rβ (Taga *et al.*, 1989). IL-6 is involved in the differentiation of Th cells to a Th2 phenotype (Rincon *et al.*, 1997). IL-6 $^{-/-}$ $CD4^+$ T cells stimulated with the T cell mitogen, ConA in the presence of wild-type APC (which could provide the IL-6 required), produced IL-4, but this IL-4 production was blocked by the addition of an anti-IL-6 monoclonal antibody (Rincon *et al.*, 1997). This suggests that IL-6 production by APCs, such as the DC, during the activation of $CD4^+$ T cells plays a major role in determining the Th phenotype. In addition, IL-6 has been shown to inhibit Th1 differentiation in an IL-4 dependent manner (Diehl *et al.*, 2000).

1.6 THE ROLE OF CHEMOKINES IN LINKING INNATE AND ADAPTIVE IMMUNITY

Chemokines, owing to their differential capacity to recruit specific leukocyte populations, ultimately determine which cells will regulate and participate in the local innate and adaptive immune responses. Chemokines belong to a supergene family of 8-10kDa protein mediators. Chemokines are sub-classified into two large (CC and CXC) and two small (C and CX3C) groups based on the number and positioning of the most highly conserved cysteines. All chemokines have at least two conserved cysteines, and all but two chemokines, have at least four. In the four cysteine group, the first two, which are located in the N-terminus, are either adjacent (CC) or else separated by at least one (CXC) or three (C3XC) non-conserved amino acids. The biological activities of chemokines are mediated by two groups of seven transmembrane receptors, CXCR and CCR (Murphy, 1996).

1.6.1 CHEMOKINE CONTROL OF DC MIGRATION

DC migration is an integral component of their sentinel function. Immature DC respond to a number of CC and CXC chemokines including MIP-1α/CCL3, MIP-1β/CCL4, MIP-3α/CCL20, MCP-1/CCL2, and RANTES/CCL5. However, each immature DC subset appears to respond to different chemokines. For example, langerhan cells, which express CCR6 and migrate selectively to MIP-3α/CCL20 and monocyte-derived DC, which express CCR1 and CCR5 migrates to MIP-1α/CCL3, MIP-1β/CCL4 (Yoshie, 2000).

Mature DC in the lymphoid tissue lose their responsiveness to most of these inflammatory chemokines by downregulating appropriate chemokine receptors on

their cell surface. These DC subsequently acquire responsiveness to MIP-3α/CCL20 and SLC/CCL21 through upregulation of CCR7 on the DC surface (Caux *et al.*, 2000; Chensue, 2001; Gunn *et al.*, 1998). This change in responsiveness ensures that mature DC home to T cell rich areas in the lymphoid tissue where MIP-3α/CCL20 and SLC/CCL21 are exclusively expressed (Willimann *et al.*, 1998). This DC migration pattern orchestrated by chemokines is vital for the induction of an adaptive immune response. Furthermore, chemokines are responsible for the rapid infiltration of DC into inflamed tissue (McWilliam *et al.*, 1996). These recruited DC in turn, participate in production of chemokines necessary for the recruitment and activation of other inflammatory cells (Rescigno *et al.*, 2000).

The nature of the chemokine response can also alter the types of leukocytes that are recruited into the sites of inflammation and ultimately result in the resolution of the inflammatory response (Zhang *et al.*, 2000). Neutralisation of MIP-2 and CXCR2 (a neutrophil specific chemokine and chemokine receptor) was shown to alleviate pulmonary inflammation by *Propionibacterium acnes* in mice (Standiford and Huffnagle, 1997b; Standiford *et al.*, 1997a). Neutralisation of SLC/CCL21 exacerbated the pulmonary inflammation due to increased numbers of mature DC, macrophages and neutrophils but decreased $CD4^+$ T cells in *P. acnes* infected lungs. The authors concluded that decreased $CD4^+$ T cell numbers were due to the fact that DC were detained in the lungs of *P. acnes* infected mice, thus preventing the antigen-specific T cell responses in local lymph nodes (Itakura *et al.*, 2001). This study illustrates the importance of chemokines in bridging the innate and adaptive immune responses.

1.6.2 THE INFLUENCE OF CHEMOKINES ON T CELL RECRUITMENT AND DEVELOPMENT

T cells that are activated in lymph nodes after CCL19/CCL21 mediated encounter with pathogen primed DC are recruited to sites of inflammation by sensing chemokine gradients at these sites. In a Th1-polarizing environment activated T cells upregulate CXCR3 and CCR5, and are attracted into sites of Th1 inflammation (Sallusto *et al.*, 1998c; Sallusto *et al.*, 1998a; Sallusto *et al.*, 1998b). Tr1 cells have also been shown to express CCR5 (McGuirk *et al.*, 2002), which suggests that these cells are recruited to sites of inflammation, possibly to counter Th1 responses. In a Th2-polarizing environment, T cells downregulate CCR7, upregulate CCR3, CCR4 and CCR8, and are attracted into sites of Th2 inflammation (Sallusto *et al.*, 1998c; Sallusto *et al.*, 1998a; Sallusto *et al.*, 1998b). The interaction between chemokines and their receptor is therefore an important step in the control of T cell migration into sites of inflammation.

The influence of chemokines on the development of T cell responses was illustrated in a gene transfer study, where the overexpression of the Th1-affliated CXC chemokine IP-10/CXCL10 in the airways of mice undergoing a dominant Th2-polarized mucosal sensitisation regimen was shown to reduce all parameters of allergic airway inflammation (Wiley *et al.*, 2001). These findings illustrate that localised expression of IP-10/CXCL10 in the lungs promoted a Th1 type response and consequently prevented a Th2 response (Wiley *et al.*, 2001). In addition, MCP-1/CCL2-deficient mice had defective Th2 responses (Gu *et al.*, 2000). The manner by which chemokines subvert the development of acquired Th1 or Th2 responses is unknown.

1.6.3 NEUTROPHIL-DERIVED CHEMOKINES

Neutrophils are an essential component of the acute inflammatory response and the resolution of microbial infection (Mayer-Scholl *et al.*, 2004). Recently, it has become clear that the contribution that neutrophils make to host defence extends beyond their phagocyte function. Neutrophils have been shown to produce a variety of chemokines such as IL-8/CXCL8 (mouse homologue is MIP-2), MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL10 (Scapini *et al.*, 2000). Through this chemokine production, neutrophils can subsequently orchestrate the chemotaxis of other leukocyte subsets including monocytes, immature DC and T lymphocyte subsets (Scapini *et al.*, 2000).

1.7 TH1 AND TH2 CELLS

The discovery of polarized subsets of $CD4^+$ T cells that differ in their cytokine secretion pattern has provided a basis for the diversity of T-cell dependent immune responses (Mosmann *et al.*, 1986). The differentiation of naïve $CD4^+$ T cells into subsets of T helper (Th) cells is a pivotal process in the immune response (Abbas *et al.*, 1996). Th1 cells produce IFN-γ and promotes cellular immunity, which is critical in the eradication of intracellular pathogens such as *Mycobacterium tuberculosis* and *Bordetella pertussis* (Mills *et al.*, 1998; North and Jung, 2004). IFN-γ activates macrophages, enhancing their ability to phagocytose and destroy microbes (Duffield, 2003). Th1 cells also promote IgG2a synthesis (Abbas *et al.*, 1996; Mahon *et al.*, 1995). In contrast, Th2 cells produce IL-4, IL-5, and IL-13 and promote humoral immunity, allergic reactions to environmental antigens, and resistance to helmintic infections (Finkelman *et al.*, 1991; Jarnicki and Fallon, 2003). During Th2 responses, IL-4 and IL-13 induce B cell switching to IgE production, whereas IL-5 is the principal eosinophil activating cytokine (Abbas *et al.*,1996). In addition to Th1 and Th2 cell types, Th0 populations have also been described that secrete combinations of Th1 and Th2 cytokines (Elson *et al.*, 1995; Firestein *et al.*, 1989). However, it has also been suggested that the Th0 population may represent a transitory precursor subset, which will eventually undergo terminal differentiation into Th1 or Th2 cells (Nakamura *et al.*, 1997).

Th differentiation is a central process in defining the adaptive immune response. It is however, regulated at many levels. As well as the critical role of DC-derived cytokine and chemokine production in T cell development (see sections 1.5, 1.6),

signature cytokine production from the Th cells themselves or other cellular sources contribute to the polarisation of a certain Th phenotype.

1.6.4 IFN-γ

IFN-γ is a key cytokine in regulating Th1 differentiation. IFN-γ is the signature cytokine of Th1 cells, and is important for stabilisation of the Th1 phenotype itself. While differentiated Th1 cells cannot be switched to produce Th2 cytokines, Th1 cells from IFN-γ-deficient mice retain the ability to produce IL-4 if restimulated under Th2-polarizing conditions (Zhang *et al.*, 2001).

Recently, the mechanistic basis for the actions of IFN-γ were uncovered. IFN-γ induces the transcription factor, T-box protein expressed in T cells (T-bet) (Afkarian *et al.*, 2002; Lighvani *et al.*, 2001). Furthermore, T-bet promotes the production of IL-12Rβ2 and IFN-γ in $CD4^+$ T cells (Lighvani *et al.*, 2001). The importance of T-bet in regulating Th1 differentiation was confirmed in T-bet deficient mice. $CD4^+$ T cell responses from T-bet deficient mice have a severe defect in Th1 polarized responses (Szabo *et al.*, 2002). In addition, T-bet deficient mice develop a spontaneous reactive airway disease similar to human asthma (Finotto *et al.*, 2002).

1.6.5 IL-4

Among the factors that regulate Th2 differentiation, IL-4 is the major known determinant. IL-4 is expressed as a 15-19 kDa protein and exists as a dimer (Yokota *et al.*, 1988). IL-4 is produced by $CD4^+$ T cells, mast cells and basophils. It induces the Th2 phenotype while suppressing the development of Th1 cells. IL-4 enhances

MHC class II expression on B cells, and promotes immunoglobulin class switching to IgG1 and IgE (Beckmann *et al.*, 1992; Yokota *et al.*, 1988).

IL-4 and the closely related cytokine, IL-13 signal through a shared surface receptor, IL-4α, which activates the transcription factor STAT-6 (Jung *et al.*, 2002; Takeda and Akira, 2000). STAT-6 deficient mice exhibit a similar phenotype to IL-4 deficient mice (Barnes, 2002).

1.7 Regulatory T cells

In addition to T cells that 'help' the immune response to eradicate a pathogen, other populations of T cells exist that down-regulate the immune response and facilitate the resolution of inflammation. The existence of suppressor T cells and their role in the maintenance of immune homeostasis was first proposed in the early 1970s (Gershon and Kondo, 1971). However, the field eventually floundered because these T cells could not be phenotyped and attempts to clone the putative suppressor cells or factors failed.

Following the re-discovery of suppressor T cells as regulatory T cells in the mid 1990s, it is now fully accepted that distinct subtypes of $CD4^+$ T cells, discriminated on the basis of phenotype or cytokine secretion, play a major role in maintenance of self tolerance, largely by their ability to suppress responses mediated by other populations of T cells. Several subsets of regulatory T cells with distinct phenotypes and distinct mechanisms of action have been described. These include, naturally occurring $CD4^+CD25^+$ T cells, type 1 T regulatory (Tr1) cells, which secrete high levels of IL-10 but not IL-4 or IFN-γ and Th3 cells, which secrete TGF-β.

$CD4^+$ $CD25^+$ natural Tr cells mature in the thymus and represent 5-10% of the peripheral $CD4^+$ T cell population and have been shown to exhibit potent immunosuppressive activity both *in vitro* and *in vivo* (Shevach, 2000). Difficulties with CD25 (expressed on all activated T cells) as a marker led to attempts to find alternative phenotypic markers for natural Tr cells. Thus, $CD4^+$ $CD25^+$ Tr cells were further characterised by the constitutive expression of several other activation markers including glucocorticoid-induced TNF receptor (GITR) (Shimizu *et al.*, 2002), OX40 (CD134) (McHugh *et al.*, 2002), L-selectin (CD62L) (Lepault and Gagnerault, 2000) and CTLA-4 (CD152) (Read *et al.*, 2000). However, the role these markers play (if any) in the suppressive abilities of $CD4^+CD25^+$ Tr cells remains poorly defined. Recently the fork head/winged helix transcription factor, Foxp3, has been proposed to be the "key to cell lineage" of naturally occurring Tr cells (Ramsdell, 2003). This transcription factor is thought to be critical in the development and function of $CD4^+CD25^+$ Tr cells as both mice and humans deficient in a functional Foxp3 protein suffer from severe autoimmune disease symptoms (Kriegel *et al.*, 2004; Ramsdell, 2003).

Although the mechanism of suppression by natural Tr cells is not fully understood, it appears to be cell contact mediated, involving CTLA-4, but a role for IL-10 and TGF-β has not been excluded (Maloy *et al.*, 2003; Read *et al.*, 2000; Shevach, 2002). Recent studies by Nakumura *et al.* have suggested a possible role for membrane-bound TGF-β in the inhibitory activity of murine $CD4^+$ $CD25^+$ Tr cells. The suppressive capacity of human $CD4^+$ $CD25^+$ thymocytes was blocked by a combination of mAb against CTLA-4 and membrane bound TGF-β *in vitro* (Nakamura *et al.*, 2001). However, $CD4^+$ $CD25^+$ Tr cells isolated from TGF-β deficient mice do not exhibit comprised suppressive activity. Furthermore, wild-type

$CD4^+$ $CD25^+$ Tr cells suppress $CD25^-$ T cells expressing a dominant-negative TGF-β receptor (Piccirillo *et al.*, 2002).

In contrast to naturally occurring $CD4^+$ $CD25^+$ Tr cells, Tr1 and Th3 cells are thought to acquire their suppressor activity through antigenic activation of $CD4^+$ $CD25^-$ T cells in the periphery. Inducible Tr cells exhibit variable expression of CD25 and other surface markers described for naturally occurring Tr cells (McGuirk *et al.*, 2002). The suppressor activity of these Tr cells is cell-cell contact independent and is mediated primarily through the actions of the anti-inflammatory cytokines IL-10 and TGF-β.

Tr1 cells were first characterised in murine models of inflammatory bowel disease as efficient suppressors of Ag-specific immune responses. T cells from OVA-TCR-transgenic mice, repeatedly stimulated with OVA and IL-10, differentiated into Tr1 cells *in vitro* (Groux *et al.*, 1997). These Tr1 cells produced IL-10, low to moderate levels of IL-5 and TGF-β, but with little or no IL-2, IL-4 or IFN-γ. Naïve $CD4^+$ T cells can be manipulated *in vitro* to generate homogenous populations of IL-10 producing Tr1 cells (Barrat *et al.*, 2002). The ability of Tr1 cells to suppress proliferative responses has been demonstrated by co-culture of naïve $CD4^+$ T cells and Tr1 cells in the presence of allogeneic APCs (Groux *et al.*, 1997). Similarly, Tr1 clones specific for filamentous haemaggultinin (FHA) from *Bordetella pertussis* suppress proliferation and cytokine production by a Th1 clone against an unrelated antigen, influenza virus haemagglutinin (HA) (McGuirk and Mills, 2000).

Th3 cells were originally identified in mice after oral tolerance induction to myelin basic protein (MBP) for their ability to suppress MBP-specific encephalomyelitis (EAE) *in vivo* in a TGF-β-dependent manner, as suppression was abrogated by injection of anti-TGF-β Ab (Chen *et al.*, 1994; Fukaura *et al.*, 1996).

Th3 cells provide help for IgA production and have suppressive properties for both Th1 and Th2 cells (Weiner, 2001). Furthermore, as TGF-β is broadly expressed and acts on multiple cell types, TGF-β-secreting Th3 cells probably have a major role in many aspects of immune regulation and homeostasis (Gorelik *et al.*, 2002).

Since regulatory Tr1 and Th3 cells arise from naïve or resting $CD4^+$ T cells in the periphery, it was highly conceivable that DC activated with an appropriate stimulus, such as certain pathogen-derived molecules, could selectively promote the induction of Tr cells. Indeed, certain pathogen-derived molecules, including FHA and adenylate cyclase toxin (CyaA) from *B. pertussis*, non-structural protein (NS) 4 from hepatitis C virus and CT have been shown to promote the induction of Tr1 cells, through their interaction with DC or other innate cells, such as macrophages. (Brady *et al.*, 2003; Lavelle *et al.*, 2003; McGuirk *et al.*, 2002; Ross *et al.*, 2004).

Pneumocystis carinii (PC) is an opportunistic pulmonary pathogen which can causes life-threatening pneumonia in immunocompromised individuals, such as AIDS patients. While an indispensable role for $CD4^+$ T cells in bacterial clearance is well established (Hanano and Kaufmann, 1999), it has also been suggested that under certain circumstances $CD4^+$ T cell-mediated inflammatory responses contribute significantly to the pneumonia pathogenesis caused by the PC fungus (Roths and Sidman, 1992). Recent studies by Hori *et al.* have suggested an important role for $CD4^+CD25^+$ Tr cells in controlling excessive T-cell mediated inflammatory responses (Hori *et al.*, 2002). Adoptive transfer of $CD4^+CD25^-$ T cells into PC infected $RAG2^{-/-}$ mice leads to a lethal pneumonia within 13 days post transfer. In contrast, transfer of $CD4^+CD25^+$ Tr cells did not induce pneumonia and prevented the development of disease induced by $CD4^+CD25^-$ T cells. Furthermore, $CD4^+CD25^-$T cells reduced PC load in the lungs while $CD4^+CD25^+$ Tr cells inhibited this immune response (Hori *et*

al., 2002). The finding that $CD4^+CD25^+$ Tr cells inhibits both pulmonary inflammation and elimination of the pathogen mediated by $CD4^+CD25^-$ T cells illustrates a dual role for Tr cells in infection, inhibiting both deleterious and protective aspects of the immune response.

1.8 MEMORY T CELLS

Memory T cells provide rapid and superior protection against pathogens that have been generated following previous encounters with the pathogen or antigen (Seder and Ahmed, 2003). Two forms of memory T cells have been identified based on functional and migratory differences; "central" T memory cells and "effector" memory cells (Sallusto *et al.*, 1999). The subset of $CCR7^+CD62L^+$ memory T cells, termed central memory T (T_{CM}) cells, outnumber the memory subset lacking these lymph node homing receptors, which are termed effector memory T (T_{EM}) cells (Sallusto *et al.*, 1999). In humans, T_{CM} cells were described as being undifferentiated, as they were unable to produce inflammatory cytokines, whereas T_{EM} cells were found to immediately initiate effector functions in response to stimulation (Sallusto *et al.*, 1999). In terms of protection against re-infection, pre-existing effector activity is very beneficial to the host by inducing an immediate response to the antigen. The distinction between T_{CM} and T_{EM} with regards to CCR7 expression is not a strict one since T_{EM} cells have the capacity to reacquire CCR7 expression upon re-stimulation (Moser *et al.*, 2004). Antigen encounter by T_{EM} cells and consequent re-expression of CCR7, may enable these cells to reach the lymph nodes via the CCL21-expressing lymphatic vessels (Brandes *et al.*, 2003).

1.9 B CELLS

B cells comprise a major component of the adaptive immune system, having the exclusive ability to produce and secrete immunoglobulins (Ig) of various forms (isotypes). B cells develop from progenitor cells in the bone marrow. B cells remain in the bone marrow for the duration of their development. The production of antigen-specific receptors results from a random rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors. Gene rearrangement occurs early in the development of the cells, before exposure to antigen, and leads to the production of a repertoire of over 10^{10} antibody specificities (Viau and Zouali, 2005).

The antigen-specific membrane receptors of B cells are Ig molecules. Igs are members of a large family of proteins designated the Ig supergene family. Many of the cell surface proteins that participate in immunological recognition, including T cell receptor (TCR), the CD3 complex, and the molecules associated with the B cell receptor (Igα, Igβ), are members of the Ig supergene family (Viau and Zouali, 2005).

The Igs themselves are constructed of a unit that consists of two heavy (H) chains and two light (L) chains. The H and L chains are composed of a series of domains, each consisting of approximately 110 amino acids. The H chains of the Ig molecules are of several classes (μ, δ, γ, α, ε). An assembled Ig molecule, consisting of one or more units of two identical H and L chain, derives its name from the H chain it possess. Thus, there are IgM, IgG, IgD, IgA and IgE antibodies.

IgM is the most versatile of the antibody classes. In the periphery, IgM can be expressed by immature, mature, and memory B cells. The presence of IgM on the surface of these cells provides a receptor for B cell activation along with the Igα and

Igβ accessory molecules (Ishihara *et al.*, 1992; Stahl and Sibrowski, 2003). While membrane bound IgM is most common, IgM plasma cells secrete polymeric IgM. Polymeric IgM is an important complement activator and thus participates in phagocytosis. IgM is second only to IgA in its contribution to mucosal immunity. In many patients with IgA deficiency, IgM adequately substitutes for IgA in mucosal protection.

IgA is the most prominent antibody found at mucosal surfaces. There are two isotypes of IgA in humans (IgA1 and IgA2) (Lehner *et al.,* 1994). IgA2 is the main component of secreted IgA (Mestecky and McGhee, 1987). IgA is an important component in the first line of defence from organisms entering by mucosal routes. While most secretory Ig is IgA, it also accounts for 10 – 15% of serum Ig. Serum IgA tends to be mostly IgA1 (Mestecky and McGhee, 1987). IgA elicits protection primarily through exclusion, binding and crosslinking of pathogens (Lehner *et al.,* 1994). A number of studies have shown that secretory IgA (sIgA) in mucosal secretions provides protection against bacteria (Czinn *et al.,* 1993; Winner *et al.,* 1991). IgA has been shown to be able to fix complement by the alternative pathway. Targets that are opsonised by IgA are removed by FcαR (CD89)-mediated phagocytosis (Woof and Kerr, 2004).

IgG is the most abundant isotype in the blood. 75% of serum Ig is comprised of IgG. IgG subclasses bind to and activate the complement with different efficiencies (Sandlie and Michaelson, 1991). IgG antibodies can be subdivided further into subclasses in mice (IgG1, IgG2a, IgG2b and IgG3) and in humans (IgG1, IgG2, IgG3 and IgG4). The interaction of CD40 ligand, expressed on the surface of activated T cells with CD40 on the surface of the B cell can stimulate B cell division (Rush *et al.*, 2002), while the provision of cytokines such as IL-4 can enhance B cell

differentiation and stimulate Ig class switching to IgG1 and IgE in the mouse (Snapper *et al.*, 1988a). In the mouse, other cytokines such as IFN-γ and TGF-β can stimulate class switching to the isotypes IgG2a (Snapper *et al.*, 1988b) and IgA (Coffman *et al.*, 1989; Sonoda *et al.*, 1989) respectively.

1.11 *BORDETELLA PERTUSSIS*

Thomas Sydendam first used the term infantum pertussis in 1670, 'pertussis' meaning a violent cough of any kind. Bordet and Gengou identified the causative organism in 1906. *Bordetella pertussis* is an obligate human pathogen and is the etiological agent of whooping cough (pertussis), an acute respiratory disease which remains a significant cause of morbidity and mortality in infants worldwide. Annually, more than 50 million cases of pertussis, including 600,000 deaths are reported worldwide (Beier *et al.*, 1996; Cherry, 1996; Kerr and Matthews, 2000). Pertussis can occur at any age, however, most cases of severe disease and the majority of fatalities occur in early infancy.

1.11.2 CLASSIFICATION AND MORPHOLOGY

B. pertussis belongs to the genus *Bordetella* in the family Alcaligenaceae. This family is comprised of several species of closely related bacteria with similar morphology. *Bordetella* are small (0.2 micron x 0.7 micron), aerobic, gram-negative coccobacilli that cause respiratory tract infections in humans and animals (Mattoo *et al.*, 2001).

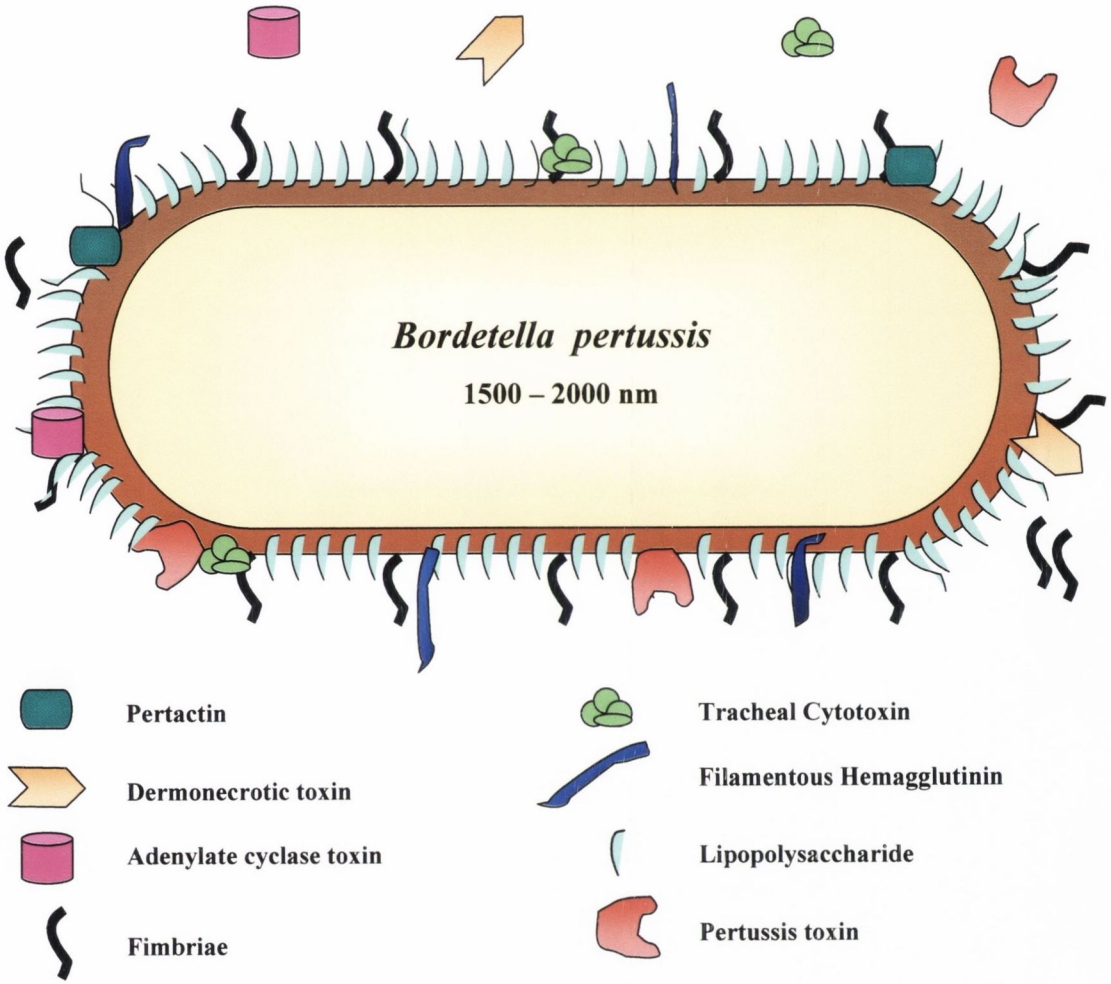


Figure 1.5 The virulence factors of *Bordetella pertussis*

1.11.3 C LINICAL MANIFESTATIONS

Pertussis is a highly infectious bacterial disease spread by droplets, with an incubation period of 7 – 10 days (range 6 – 20 days). Transmission is thought to occur primarily by respiratory droplets. Infection is characterised by colonisation of ciliated respiratory epithelia in the trachea and bronchia. The clinical course of pertussis is divided into three stages. The onset of disease is gradual, the initial catarrhal stage is marked by an irritated cough and slight fever, lasting 1 – 2 weeks. As the frequency of the cough increases, the disease enters the classic stage, paroxysmal stage approximately 7 – 14 days after the initial onset of cough. During this stage, periods of violent coughing occur, followed in some cases by a sudden and rapid inspiration of air through the swollen upper airways, resulting in the characteristic 'whoop'. The severity of the disease at this stage is age dependent and is most dramatic in children less than one year of age. The duration of the paroxysmal stage varies from 1 to 6 weeks. The final phase of the infection is marked by decreased paroxyms, possible cough and secondary bacterial infections. (Hewlett, 1997).

1.11.4 V IRULENCE FACTORS OF *B. PERTUSSIS*

Strategies employed by bacterial pathogens, the number and nature of virulence factors required to effect those strategies, and the regulatory systems that control virulence gene expression are the result of co-evolution between bacteria and their hosts (Cotter and DiRita, 2000). Bacteria have evolved the ability to resist host defence mechanisms and to survive and multiply within several different intracellular

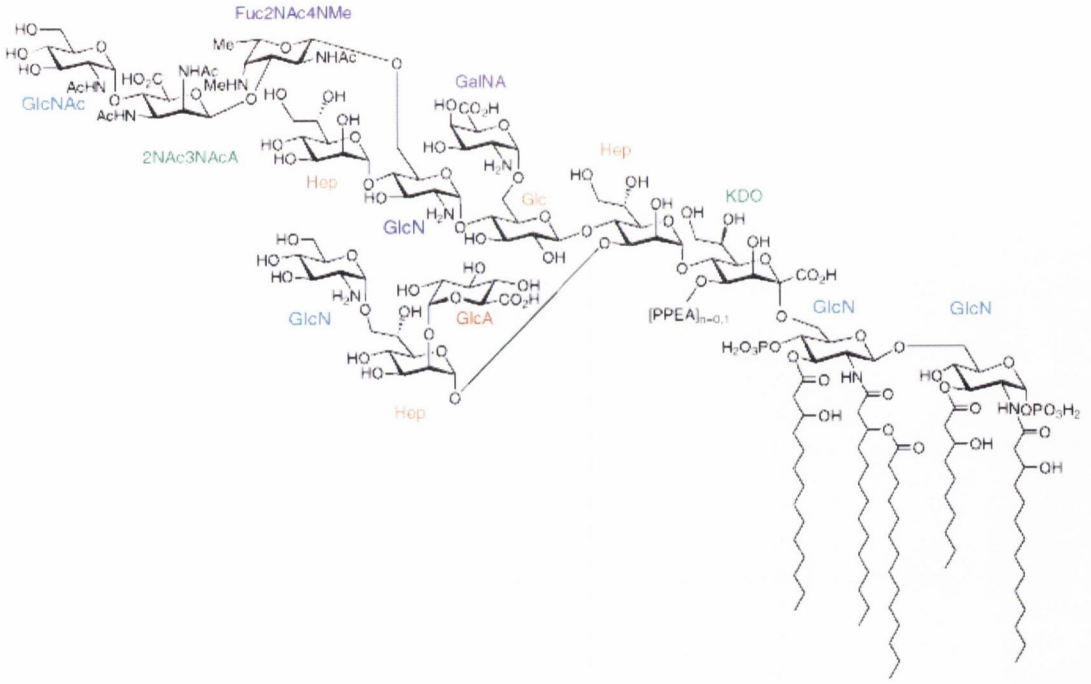
and extacellular environments. These abilities require the functions of many different virulence factors.

1.11.4.1 LIPOPOLYSACCHARIDE

The lipopolysaccharide (LPS) contained in the outer membranes of *Bordetella* species, like LPS from other gram-negative bacteria is pyrogenic, mitogenic, toxic, and can activate pro-inflammatory cytokine production from macrophages (Amano *et al.*, 1990; Amano *et al.*, 1991; Ayme *et al.*, 1980; Watanabe *et al.*, 1990). The LPS molecule of *B. pertussis* is smaller than many other bacterial LPS structures and is therefore, often referred to as lipooligosaccharide (LOS) (Preston and Maskell, 2001). A typical LPS molecule from a gram-negative bacterium generally is comprised of a lipid A, core oligosaccharide and a long polysaccharide O antigen. The structure of LOS differs in that it lacks the O antigen (Preston *et al.*, 1999). *B. pertussis* actually produces 2 types of LPS, designated A and B (Peppler, 1984). *B. pertussis* LPS resolves as 2 distinct bands on silver stained sodium-dodecyl sulphate-polyacrylamide (SDS) gels (Peppler, 1984). The faster migrating moiety, band B, consists of a lipid A molecule linked via a single ketodeoxyoctulosonic acid (KDO) residue to a branched oligosaccharide core structure (Caroff *et al.*, 1990; Caroff *et al.*, 2002; Lasfargues *et al.*, 1993; Preston and Maskell, 2001). The slower migrating moiety (band A) consists of band B linked to a trisaccharide (Caroff *et al.*, 1990; Caroff *et al.*, 2002; Lasfargues *et al.*, 1993; Preston and Maskell, 2001).

The construction of defined LPS mutants (by the deletion of *wlb* locus which resulted in the loss of the membrane-distal polysaccharide, leaving LPS molecules consisting of lipid A and core oligosaccharide) has provided evidence that wild-type

(a)



(b)

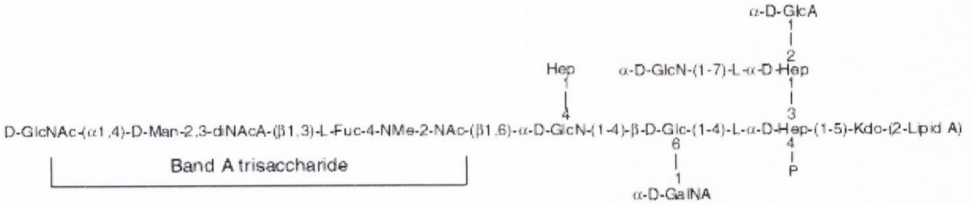


Figure 1.6 (a) The chemical structure and (b) its abbreviated formula of *Bordetella pertussis* LPS

of LPS is required for colonisation and survival of Bordetella *in vivo* (Preston and Maskell, 2001). The *B. pertussis* LPS mutant was found to be defective in colonisation of the nasal cavity when compared to the wild-type (Harvill *et al.*, 2000; Preston and Maskell, 2001).

LPS is present in varying concentrations in most whole cell pertussis vaccines (Pw) and it has been demonstrated that removal of LPS from pertussis vaccine preparations resulted in decreased efficacy (Bannatyne and Jackowski, 1987). In combination with other toxins, in particular pertussis toxin (PT), LPS is responsible for the reactogenicity and adjuvant effect of whole cell pertussis vaccine (Pw) through the induction of IL-1β (Donnelly *et al.*, 2001; Loscher *et al.*, 2000; Loscher *et al.*, 1998). Although LPS plays a major role in the systemic and neurological responses to infection and vaccination, it can be beneficial to the host in bacterial clearance by stimulating the production of IL-12 which enhances the activation of IFN-γ secreting NK cells, which in turn promote the differentiation of naïve T cells to a polarized Th1 subtype (Byrne *et al.*, 2004; Mahon *et al.*, 1996).

1.11.4.2 TRACHEAL CYTOTOXIN

Tracheal cytotoxin (TCT), a disaccharide-tetrapeptide monomer of peptidoglycan that is released by growing *B. pertussis*, induces cytopathology in hamster tracheal ring organ cultures (loss of cilia, disruption of tight junctions, and extrusion of ciliated cells from the respiratory surface) similar to the pathology observed during pertussis disease (Cookson *et al.*, 1989a). TCT, a 921-Da molecule, was first identified in 1982 by Goldman and co-workers (Goldman *et al.*, 1982). The primary structure of TCT is N-acetylglucosaminyl-1,6-anhydro-N-

acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine (Cookson *et al.*, 1989a). The structure of TCT places it in the muramyl peptide family, a group of structurally related molecules that are responsible for a diverse range of biological activities.

Neisseria gonorrhoeae, which also damages human ciliated cells during gonococcal infection of fallopian tube mucosa, releases a 921-Da molecule that is chemically identical to TCT (Sinha and Rosenthal, 1980). More recently, TCT was isolated from the luminous, gram-negative bacterium, *Vibrio fischeri* (Koropatnick *et al.*, 2004). However, the release of TCT is not a general property of gram-negative bacteria, despite the fact they share a common peptidoglycan composition (Rosenthal *et al.*, 1987).

B. pertussis TCT causes ciliostasis, ciliated cell destruction within cultured hamster respiratory epithelia and can also inhibit DNA synthesis in isolated cultured hamster tracheal epithelial (I) cells (Cookson *et al.*, 1989b). TCT inhibition of I proliferation may reflect a secondary effect of TCT on the capacity of respiratory epithelium to regenerate the lost ciliated cell population (Cookson *et al.*, 1989a).

IL-1 has been reported to be involved in TCT-mediated toxicity (Heiss *et al.*, 1993a). Recombinant IL-1α causes TCT-like damage to the respiratory epithelium (Heiss *et al.*, 1993a). IL-1 inhibits DNA synthesis by I cells and generates pertussis-like destruction of epithelial cells in hamster tracheal organ culture (Heiss *et al.*, 1993a). Furthermore, TCT stimulates IL-1α production by respiratory epithelial cells. The IL-1α produced remains intracellular, consistent with the observations that the effects of TCT cannot be blocked using either anti-IL-1α antibodies or the IL-1 receptor antagonist (Heiss *et al.*, 1993a). The ability of exogenously added recombinant IL-1 to duplicate TCT effects is not inconsistent with an intracellular IL-1 function, since exogenous IL-1 has been shown to be translocated to the nucleus

(Mizel *et al.*, 1987). Induction of nitric oxide synthase (NOS) in the respiratory epithelium has been reported to be upstream of intracellular IL-1 production in the generation of TCT toxicity (Heiss *et al.*, 1994).

Infiltration of neutrophils, which phagocytose and kill invading microorganisms represents an important immune defence mechanism. It has been demonstrated that TCT is toxic for human neutrophils (Cundell *et al.*, 1994). TCT may therefore contribute to the survival of *B. pertussis* within the airways *in vivo*. TCT and LPS have also been found to be highly synergistic in the induction of IL-1α, inducible NOS, nitric oxide (NO) and inhibition of DNA synthesis when added to I cells (Flak *et al.*, 2000).

Recently, TCT was shown to activate an ancient innate defence pathway in the fruit fly, *Drosophila melanogaster*. Insects depend solely on innate immune responses to survive infection. In *Drosophila*, the IMD pathway (named after 'immune deficient' mutants) is required for antimicrobial gene expression in response to gram-negative bacteria (Lemaitre *et al.*, 1995). Kaneko and co-workers demonstrated that highly purified LPS did not stimulate this pathway. However, the IMD pathway was very sensitive to TCT (monomeric) and polymeric gram-negative peptidoglycans (Kaneko *et al.*, 2004). Activation of the IMD pathway was found to require the diaminopimelic acid residue of gram-negative peptidoglycans.

1.11.4.3 PERTUSSIS TOXIN

Pertussis toxin (PT) is a major colonising factor and an exotoxin, which remains cell bound as well as released into the extracellular environment. PT is a 105 kDa hexamer with a typical AB architecture, consisting of an A subunit and a

complex pentamer B subunit (Saukkonen *et al.*, 1992). The B subunit binds to host cell surfaces and the A subunit is an ADP ribosyltransferase (Tamura *et al.*, 1982). The A monomer consists of the enzymatically active S1 subunit and sits on top of the B oligomer which is a ring like structure formed by the remaining subunits (S2-S5) in a ratio of 1:1:2:1 (Saukkonen *et al.*, 1992). The A and B subunits share significant homology with other bacterial toxins such as cholera toxin (CT) of *Vibrio cholerae*, shiga toxin of *Shigella dysenteriae*, and the labile toxin of *Escherichia coli* (LT).

PT exhibits enzymatic activity as an NAD-dependent ADP-ribosyltransferase (Katada *et al.*, 1983). The enzyme substrate for PT is the regulatory GTP-binding protein, Gi, which exerts inhibitory activity on membrane associated eukaryotic adenylate cyclase. PT causes irreversible inactivation of Gi α subunits, thereby blocking inhibition of adenylate cyclase activity leading to unchecked □amp production. Other GTP-binding regulatory membrane proteins are also inactivated by PT, such that a variety of receptors become uncoupled from inhibitory input. The result is an extensive disruption of intracellular signalling pathways (Chaffin *et al.*, 1990)

PT interacts with many types of mammalian cells and has multiple biological effects, which include affecting the recirculation of leukocytes (Spangrude *et al.*, 1984), altering the response of neutrophils to N-formylated peptides that are chemoattractants (Bokoch and Gilman, 1984), and inhibiting the migration of macrophages (Meade *et al.*, 1984a). Using mouse and rat models, PT has been shown to inhibit chemotaxis and migration of neutrophils, monocytes/macrophages and lymphocytes (Brito *et al.*, 1997; Meade *et al.*, 1984b). PT has also been suggested to function as an adhesin, involved in the adherence of *B. pertussis* to human

macrophages and ciliated respiratory epithelial cells (Relman *et al.*, 1990; Tuomanen *et al.*, 1983).

Some of the clinical symptoms of pertussis can be reproduced in experimental animals using purified PT (Pittman, 1984). Chemically or genetically detoxified forms of the toxin are an essential component of acellular pertussis vaccines. PT also has immunomodulatory activity enhancing T cell and antibody responses to co-injected antigens (Ryan *et al.*, 1998). Furthermore, PT enhances LPS-induced IL-12 production from DC and macrophages (Hou *et al.*, 2003).

1.11.4.7 FILAMENTOUS HAEMAGGLUTININ

A critical step in *B. pertussis* infection is the attachment of the pathogen to host cells. Filamentous Haemagglutin (FHA), is regarded as the major adhesin of *B. pertussis* (Locht *et al.*, 1993). It is a 220 kDa surface-associated protein secreted into the extracellular environment to facilitate adherence to ciliated respiratory epithelial cells, thereby, initiating the infection. As the name suggests, FHA is a filamentous structure, 2 nm wide and 45 – 50 nm long (Arai and Sato, 1976; Makhov *et al.*, 1994). Makhov and co-workers proposed that FHA exists as a polypeptide chain folded into a monomeric hairpin (Makhov *et al.*, 1994). FHA is initially synthesised as a 367 kDa precursor, FhaB, which is modified at its N terminus and cleaved at its C terminus to form the mature 220 kDa FHA protein (Jacob-Dubuisson *et al.*, 1996; Renauld-Mongenie *et al.*, 1996).

FHA has an Arg-Gly-Asp (RGD) motif, which stimulates adherence to monocyte/macrophages and possibly other leukocytes via the leukocyte response integrin/integrin associated protein (LRI/IAP) complex via CR3 (Ishibashi *et al.*,

1994). Ligation of CR3 to macrophages induces phagocytosis but does not stimulate intracellular killing of the bacteria due to suppressive effects of the toxins of the bacteria. This facilitates intracellular survival and persistence of *B. pertussis* in the lungs.

FHA has been implicated in the suppressed T cell responses in the lung during acute infection (McGuirk *et al.*, 2000). McGuirk and Mills (2000) demonstrated that interaction of FHA with receptors on macrophages results in the suppression of the proinflammatory cytokine, IL-12, via an IL-10 dependent mechanism (McGuirk and Mills, 2000). FHA has also been shown to exert an immunomodulatory effect on immune responses to an unrelated pathogen, i.n administration of FHA to mice infected with influenza virus suppressed the virus-specific Th1 response in the local lymph nodes (McGuirk *et al.*, 2000). FHA has also been shown to promote the induction of Tr1 cells, through their interaction with DC (McGuirk *et al.*, 2002).

Oda and co-workers have demonstrated that active or passive immunisation with FHA protects mice against lethal infection with virulent *B. pertussis* and reduces the bacterial load in the lungs of infected mice (Oda *et al.*, 1984). Serum antibodies to FHA have been detected in children following immunisation with acellular pertussis (Pa), or whole cell (Pw) vaccines and in pertussis convalescent children (Sato *et al.*, 1984).

1.11.4.4 ADENYLATE CYCLASE TOXIN

Adenylate cyclase toxin (ACT or CyaA) is an important invasive toxin produced by *B. pertussis*. It is a bifunctional calmodulin-sensitive adenylate cyclase/hemolysin protein. ACT is synthesised as a protoxin monomer of 1706 amino

acids. The ACT pro-toxin is catalysed by CyaC mediated palmitoylation (Hackett *et al.*, 1994; Mock and Ullmann, 1993). The catalytic activity is associated with the adenylate cyclase domains located within the N terminal 400 amino acids of ACT (Glaser *et al.*, 1989; Glaser *et al.*, 1988). The 1300 amino acid long C-terminal domain mediates the delivery of the catalytic domain into the cytoplasm of cells and possesses low □amp□lytic activity for sheep red blood cells (Ehrmann *et al.*, 1991; Ehrmann *et al.*, 1992; Rogel *et al.*, 1991).

ACT can enter a number of eukaryotic cell types (Ladant and Ullmann, 1999). Once inside the cell, ACT is activated by calmodulin and catalyses the production of supraphysiological concentrations of □amp (Confer and Eaton, 1982; Confer *et al.*, 1984; Farfel *et al.*, 1987; Farfel and Hanski, 1985).

ACT has been shown to induce apoptosis in cultured murine macrophages (Khelef *et al.*, 1993). ACT-deficient *B. pertussis* mutants are defective in their ability to cause lethal infections in infant mice (Goodwin and Weiss, 1990). Furthermore, significantly fewer inflammatory cells, particularly PMNs are recruited to the lungs in response to infection with the ACT-deficient *B. pertussis* mutants compared to the wild-type *B. pertussis* (Khelef *et al.*, 1994). Recently, ACT has been reported to enhance TLR ligand induction of IL-10 and suppress IL-12 production from DC and helps to promote the induction of Tr1 cells (Ross *et al.*, 2004).

1.11.4.5 PERTACTIN

Pertactin, a 69 kDa non-fimbrial outer membrane protein has been proposed to play a role in the attachment as the protein contains an Arg-Gly-Asp (RGD) tripeptide motif as well as proline rich and leucine rich repeats, motifs commonly present in

molecules that form protein-protein interactions (Leininger *et al.*, 1991). However, it has also been reported that the RGD motif of pertactin is not involved in cell binding (Everest *et al.*, 1996). Mutant *B. pertussis* strains, which do not express pertactin, were 30 – 40% less adherent to HeLa and CHO cells, compared with the wild-type strain (Leininger *et al.*, 1991). In contrast, Roberts *et al.,* reported that pertactin mutant strains of *B. pertussis* did not differ from the wild-type strain in its ability to adhere to or invade Hep2 cells in vitro or to colonise the respiratory tracts of mice *in vivo* (Roberts *et al.*, 1991). A number of studies have demonstrated that pertactin is an immunoprotective antigen when used in subunit and live vaccines to protect mice from respiratory challenge with virulent *B. pertussis* (Roberts *et al.*, 1993; Romanos *et al.*, 1991; Shahin *et al.*, 1995; Strugnell *et al.*, 1992).

1.11.4.8 FIMBRIAE

Fimbriae, also known as pili and agglutinogens, are long filamentous protruscans, which extend from the bacterial cell surface. *B. pertussis* produces two distinct types of fimbriae, serotype 2 and serotype 3 (also called serotype 6). These are constructed using major subunits expressed from *fim2* and *fim3* respectively, and the minor fimbrial subunit is encoded by *fimD* (Locht *et al.*, 1992; Willems *et al.*, 1993). Binding of fimbriae to monocytes results in the activation of complement receptor 3 (CR3), the monocyte receptor for FHA, suggesting that synergy between fimbriae and FHA may occur during infection (Menozzi *et al.*, 1991).

1.11.4.9 REGULATION OF VIRULENCE GENES

Expression of the primary virulence factors with the exception of TCT, is regulated by the BVG (Bordetella virulence gene) locus, which encodes BvgAS in response to environmental changes (Cotter and DiRita, 2000). BvgAS is a two component signal transducing system. When the bacterium is in the Bvg^+ phase, virulence factors necessary for infection are expressed, but in the Bvg^- phase, the bacterium is not pathogenic (Cotter and DiRita, 2000).

1.12 PERTUSSIS VACCINES

In the prevaccine era, child mortality rates for pertussis were similar to those of diphtheria and scarlet fever (Grant and Cherry, 2002). In the United States, epidemic cycles occurred every 2 – 5 years, with average attacks reported to be 157/100,000 (Cherry, 1996). With the introduction of whole cell pertussis vaccine (Pw), into general use, the incidence of the disease fell dramatically. However, vaccination has not brought eradication of the disease. Although immunisation has had a dramatic effect in reducing disease burden, pertussis remains an endemic disease in adults. The persistent circulation of *B. pertussis* in adults provides a source for epidemics in susceptible infants and children (Grant and Cherry, 2002).

1.12.1 W HOLE CELL PERTUSSIS VACCINE

Attempts to develop vaccines against *B. pertussis* infection were initiated soon after the first isolation of the organism almost 100 years ago (Cherry, 1996). The first

vaccines were prepared from bacteria harvested in cultures on Bordet Gengou agar or similar media and killed by exposure to heat or chemical agents. In 1925, Madsen described the first use of whole cell pertussis vaccines and vaccination was shown to successfully contain two pertussis outbreaks in the Faroe Islands (Madsen, 1933; Madsen, 1925). However, Madsen also reported two deaths within 48 hours of immunisation (Madsen, 1933) (Kendrick and Eldering, 1935). The bacterial content of their vaccine was regulated by opacity measurement and potency was controlled by the development of a mouse protection assay involving intracerebral challenge (Kendrick and Eldering, 1939; Kendrick *et al.*, 1947). Applications of these methods were effective in producing protective vaccines but there were growing concerns regarding the reactogenicity. The improved Pw was combined with diphtheria and tetanus toxoids to produce the diphtheria tetanus pertussis (DTP) vaccine available today (Cherry, 1996). Studies of vaccine efficacy have demonstrated the efficacy of Pw to range from 81 to 93%, demonstrating that the vaccine is highly effective against severe pertussis (Church, 1979).

Due to the reactogenicity associated with the DTP vaccine, vaccine manufacturers manipulated the culture techniques in an attempt to lessen toxicity. In Sweden and the U.S in the 1970s, these attempts led to an ineffective vaccine (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Romanus *et al.*, 1987)

In 1974, Kulenkampff examined the medical records of 36 children who were presented at the Hospital for Sick Children, London, between 1961 and 1972 with neurological illness (defined as convulsions, infantile spasms, unconsciousness) thought to be associated with pertussis immunisations. Of the 36 cases, 2 children died within 6 months of onset, 4 experienced complete recovery, and the remainder experienced paralysis, epilepsy and/or moderate to severe mental retardation

(Kulenkampff *et al.*, 1974). Kulenkampff's findings were made public and were soon followed by additional reports (Stewart, 1977) documenting adverse reactions. These reports had drastic ramifications on vaccine uptake. In 1974 – 1978, vaccine acceptance rates for pertussis fell from around 80% to 31% in the U.K (Fenichel, 1983). During the 1977-1979 pertussis outbreak, there was 4 times the number of cases of that documented for the 1974 – 1975 outbreak with the highest rate occurring in children aged 1 –4 years (Pollard, 1980).

1.12.2 A DVERSE REACTIONS ASSOCIATED WITH PW

Common, minor reactions observed following administration of Pw, include local reactions (which occur in approximately 50% of recipients), such as redness, swelling and pain at the site of injection, and systemic reactions, such as fever, irritability and drowsiness (Cody *et al.*, 1981). More severe adverse reactions including seizures have been reported following pertussis vaccination. The majority of seizures are febrile and the subject often had a family history of seizure disorders (Blumberg *et al.*, 1993). Some rare debilitating and sometimes fatal reactions have been reported following Pw administration, such as encephalopathy, infantile spasms, Reye syndrome and sudden infant death syndrome (SIDS). The National Childhood Encephalopathy Study conducted between 1976 and 1979 is the largest DTP vaccine-encephalopathy study undertaken to date and also provided a 10 year follow-up report (Miller *et al.*, 1993; Miller *et al.*, 1989). The authors of this study concluded that there may be, on rare occasions, an association between the development of severe acute neurological illness and DTP administration. Recent studies in a murine model by Mills and co-workers have shown that it is possible to induce fever and seizure-

like behavioural changes in mice following parenteral vaccination with Pw or *B. pertussis* LPS and that these effects appear to be mediated (at least in part) by IL-1β (Armstrong *et al.*, 2003; Donnelly *et al.*, 2001; Loscher *et al.*, 2000). Importantly, the amount of LPS in Pw has been shown to have statistically significant associations with the frequency of fever after vaccination (Baraff *et al.*, 1989).

1.12.3 A CELLULAR PERTUSSIS VACCINES

Concerns regarding the safety of Pw motivated the development of a new generation of acellular pertussis vaccines (Pa), which are prepared with highly purified antigens from *B. pertussis* (Greco *et al.*, 1996; Olin *et al.*, 1997). The work of Sato and colleagues facilitated the identification and selective extraction of individual *B. pertussis* antigenic constituents that induced an immune response in the host (Sato and Arai, 1972; Sato *et al.*, 1974; Sato *et al.*, 1973; Szoke *et al.*, 2004). The first Pa vaccine composed of PT and FHA, was introduced in Japan in 1981 (Sato *et al.*, 1984). Today, all of the currently available Pa contain PT (chemically or genetically detoxified) alone or PT in combination with one or more purified proteins of *B. pertussis*, including FHA, pertactin and fimbrial agglutinogens. The efficacy of Pa assessed in the extensive trials performed from the mid-eighties and the nineties has ranged from 71% to 86% depending upon the study designs and case definitions (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Heininger *et al.*, 1998; Trollfors *et al.*, 1995).

1.12.4 A DVERSE EFFECTS ASSOCIATED WITH PA

Pa combined with tetanus and diphteria toxoid vaccines (DTPa) have consistently been shown to be less reactogenic than DTPw (Decker *et al.*, 1995; Halperin *et al.*, 1995; Pichichero *et al.*, 2000; Pichichero and Passador, 1997). However, it is now well established that rates of local reactions increase with each subsequent dose of DTPa (Halperin *et al.*, 1995; Noble *et al.*, 1987; Pichichero *et al.*, 2000). The main side affect associated with Pa is swelling of the entire injected thigh after a fourth consecutive dose of 2- and 3- component DTPa vaccines (Rennels *et al.*, 2000; Schmitt, 1996; Schmitt, 1997).

1.13 IMMUNITY TO *B. PERTUSSIS*

Very little is definitively known about the human immune response to primary *B. pertussis* infection in most individuals because it generally has been studied in individuals who were previously vaccinated (Mills, 2001). Animal models have therefore, proved very valuable in advancing our current understanding of immunity to *B. pertussis*. Respiratory challenge by aerosols or intranasal administration of the bacteria has been used extensively in studies attempting to determine the mechanisms of immunity to *B. pertussis* (Mahon *et al.*, 1997; Pittman *et al.*, 1980; Sato *et al.*, 1980). Recovery from primary *B. pertussis* infection provides long lasting protective immunity against subsequent disease, however, the precise mechanisms employed by the immune response, which confer this protection and contributes to bacterial clearance remain to be fully elucidated. This lack of insight into the basis of

protective immunity against disease and infection has hampered attempts to improve pertussis vaccines.

1.13.1 CELL-MEDIATED IMMUNITY

Recently, it has become apparent that *B. pertussis* can exploit both intracellular and extracellular niches during infection (Mills, 2001). In 1969, Cheers and Gray demonstrated that *B. pertussis* had an intracellular phase and suggested that cellular immunity may be required to eliminate intracellular bacteria (Cheers and Gray, 1969). Since this finding, *B. pertussis* has been shown to invade and survive within many mammalian cell types including macrophages, PMNs and respiratory epithelial cells (Bassinet *et al.*, 2000; Hazenbos *et al.*, 1994; Hellwig *et al.*, 1999; Saukkonen *et al.*, 1991; Steed *et al.*, 1992; Weingart and Weiss, 2000). The ability of *B. pertussis* to invade and survive within macrophages may be an important feature of the protective immune response against *B. pertussis* (Mills, 2001). Furthermore, pertussis has been documented in adults and children with HIV and macrophages recovered from the BAL fluid from an AIDs patient was shown to contain viable *B. pertussis* (Bromberg *et al.*, 1991). The dual extra- and intra- cellular location of *B. pertussis* is consistent with a role for cellular as well as humoral responses for bacterial elimination from the respiratory tract.

The first direct evidence of the importance of T cells in immunity to *B. pertussis* was provided by the demonstration that athymic (nude) mice or severe combined immunodeficient (SCID) mice failed to clear a *B. pertussis* infection whereas normal BALB/c mice resolved the infection after 35 days (Mills *et al.*, 1993). The protective role of T cells was further confirmed by adoptive transfer experiments.

$CD4^+$ T cells from immune convalescent mice or FHA-specific $CD4^+$ T cell line but not $CD8^+$ T cells confer a high level of protection to athymic or sub-lethally irradiated recipient mice in the absence of detectable antibody responses (Mills *et al.*, 1993). The protective T cells were shown to secrete IFN-γ but not IL-4, a cytokine profile characteristic of Th1 cells. Further evidence for a role for $CD4^+$ T cells was provided by the demonstration that CD4 deficient mice could not be protected by intranasal immunisation with inactivated bacteria (Leef *et al.*, 2000).

The signature Th1 cytokine, IFN-γ, was detected in the sera and bronchoalveolar lavage fluid (BAL) of *B. pertussis* infected mice (Torre *et al.*, 1993). Studies using IFN-γ receptor (IFN-γR) deficient and IFN-γ deficient mice have demonstrated that IFN-γ plays a key role in protection (Barbic *et al.*, 1997; Mahon *et al.*, 1997). In these studies, IFN-γ was shown to be critical in the control of bacterial dissemination, pathology and bacterial clearance after respiratory challenge infection of adult mice (Barbic *et al.*, 1997; Mahon *et al.*, 1997). IFN-γ and IL-12 were shown to enhance resistance of mice to infection, either by direct activation of alveolar macrophage by IFN-γ, or through IL-12-induced generation of Th1 cells (Torre *et al.*, 1996) .

A role for cellular immunity in vaccine-induced protection has also been identified. An examination of T cell responses in children immunised with Pw or Pa suggested that these vaccines induced distinct sub-populations of $CD4^+$ T cells. Several studies demonstrated that Pw selectively induced Th1 cells and that Pa generated T cells with a Th0 or mixed Th1/Th2 profile (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998). However, there is also evidence to suggest that the Th1/Th2 dichotomy with Pa and Pw in mice may not be so clear cut. *B. pertussis*-specific T cell responses in mice immunised with Pa shifted to a mixed Th1/Th2

profile after respiratory infection with *B. pertussis* and an addition of IL-12 to a Pa formulation resulted in the induction of IFN-γ secreting T cells (Mahon *et al.*, 1996). The addition of IL-12 was also shown to enhance Th1`cytokine production and augment the protective capacity to a level of a potent Pw (Mahon *et al.*, 1996), providing further evidence for a protective role for Th1 cells (Mills, 2001).

1.13.2 HUMORAL IMMUNITY TO *B. PERTUSSIS*

The mechanism of antibody-mediated immunity to *B. pertussis* is poorly understood. Phagocytosis and subsequent killing by macrophages represents a potent immune mechanism for clearing bacteria from the respiratory tract of the infected host. Antibody-mediated phagocytosis is a key mechanism for *B. pertussis*-mediated uptake by phagocytic cells (Hellwig *et al.*, 2001; Muraoka *et al.*, 1999; Rodriguez *et al.*, 2001). Antibody may also confer protection by other mechanisms, such as complement-mediated killing, blocking of bacterial adherence and antitoxin activity.

For certain vaccines such as those for *Haemophilus influenza* type B (Hib), diphtheria and polio vaccines, it is possible to define the level of protection by the identification of circulating antibodies. In the case of pertussis, it has not been possible to define a level of antibodies against a single antigen or combination of antigens that would predict protection (Mills, 2001).

In humans, passive immunisation with *B. pertussis*-specific anti-sera reduced the severity of the disease in infected individuals (Granstrom *et al.*, 1991). Passive immunisation experiments in mice have demonstrated that antibodies against PT, FHA, Pertactin, Fimbrae and LPS can confer a level of protection (Bruss *et al.*, 1999). Antibodies against FHA conferred the poorest protection in the respiratory challenge

model (Mills *et al.*, 1998). In a direct comparison of protective efficacy of individual antigens in mice, active and passive immunisation experiments demonstrated that PT and pertactin were the most protective antigens. This study also reported increasing protection was demonstrated with multi-component vaccines (Mills *et al.*, 1998). The superior protection efficacy of pertactin may be explained by the recent report that it is the only antigen in currently available Pa vaccines that induces opsonising antibodies (Hellwig *et al.*, 2003).

In clinical trials, serological studies reported high antibody titres against each of the antigenic components contained in Pa regardless of the vaccine efficacy (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Olin, 1997; Simondon *et al.*, 1997; Trollfors *et al.*, 1995). Immunisation with Pw also induces the antibody titres against several individual *B. pertussis* antigens, although these were more variable, in particular against PT, and this appears to reflect the variable presence of these components in the vaccine (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Olin, 1997; Simondon *et al.*, 1997; Trollfors *et al.*, 1995).

Immunoglobulin (Ig) deficient mice fail to clear *B. pertussis* suggesting that antibodies have a role in the clearance of *B. pertussis* (Mahon *et al.*, 1997). Furthermore, immunisation with Pa or Pw induced only a marginal protective effect in Ig deficient mice, and defective T cell responses as well as antibody production were also reported (Mills *et al.*, 1998). Leef and co-workers demonstrated that intranasal immunisation of Ig deficient mice with formal-fixed *B. pertussis* resulted in partial protection, which was restored to full protection after transfer of *B. pertussis* immune B cells (Leef *et al.*, 2000). These results suggest an alternative role for B cells in protection against *B. pertussis* other than the production of antibody.

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. AMMONIUM CHLORIDE LYSIS SOLUTION

0.77 g Ammonium Chloride (NH_4Cl)

Dissolved in 100 ml ddH_2O and pH adjusted to 7.2

2.1.2 Bordet Gengou Medium

250 ml ddH_2O

2.5 ml Glycerol

7.5 g Bordet Gengou Agar

The medium was autoclaved, allowed to cool and supplemented with 1 ml cephalexin and 50 ml of pre-warmed sterile defibrinated horse blood.

2.1.3 Cephalexin

0.1 g Cephalexin dissolved in ddH_2O and filter sterilised

2.1.4 1% CASEIN

6.0g Sodium Chloride (NaCl)

10 g Casamino Acids

Dissolved in 1L of ddH_2O, pH adjusted to 7.2 and autoclaved at 115°C for 20 min

2.1.5 Cell Culture Medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Biowest) was supplemented with 8% heat inactivated (56°C for 30 min) foetal calf serum (FCS), 100mM L-Glutamine (Gibco), 100 μg/ml streptomycin (Biowest). Complete RPMI was used to culture all cells isolated from murine spleens and lymph nodes.

2.1.6 ELISA Stopping Solution (1M H_2SO_4)

26.74 mls 18 M H_2SO_4

473.26 mls dH_2O

2.1.7 FACS Buffer

2% FCS

0.1% Sodium azide

Made up in PBS.

2.1.8 FACS Blocking Buffer

50% FCS

50% FACS Buffer

2.19 Formalin Buffered Saline

10% Formalin

0.9% saline

Made up in dH_2O

2.1.10 Phosphate-Buffered Saline (PBS)

80 g Sodium chloride (NaCl, 1.4M)

11.6 g Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)

2 g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)

2 g Potassium chloride (KCl, 0.03M)

Dissolved in 1L of ddH_2O and adjusted to pH 7.0

2.1.11 Phosphate Citrate Buffer

20.38g Citric Acid

73.8g di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_20$)

Made up to 2L with dH_2O, pH 5.0, stored at 4^oC.

2.1.12 Stainer and Scholte Medium

Basal Medium

10.72 g L- Glutamic acid (monosodium salts) (Sigma)

0.24 g L-Proline (Sigma)

2.5 g NaCl

0.5 g KH_2PO_4

0.2 g KCL

0.3 g Magnesium chloride ($MgCl_2.6H_2O$) (Sigma)

0.4 g Calcium chloride ($CaCl_2.2H_2O$) (Sigma)

1.525 g Tris

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

Supplement

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

0.5 g Ferrous sulphate ($FeSO_4.7H_2O$) (Sigma)

0.6 g Ascorbic acid (Sigma)

0.7 g Nicotinic acid (Sigma)

1.0 g Glutathione (Sigma)

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

2.2 METHODS

2.2.1 ANIMALS

Specific pathogen-free BALB/c (H-2^d), C3H/HeN and C3H/HeJ (H-2^k) mice were purchased from Harlan UK Ltd., Bicester, Olac, UK and were maintained according to the regulations and guidelines of the Irish Department of Health. Experiments were performed under license from the Department of Health and with the approval of the university ethics committee. All mice used were female 6-12 weeks old at the initiation of each of the experiments.

2.2.2 CULTURING OF CELL LINES

The J558 cell line that had been established by cloning the mouse gene for GM-CSF into a mammalian expression vector transfected into the plasmacytoma line V53-Ag (Karasuyama *et al.*, 1990) was obtained from Dr. Nathalie Winters (Pausteur Institute, France). The first two passages of J558 cell line were grown in selection medium i.e. RPMI supplemented with geneticin (G418 sulphate; Gibco BRL). After the second passage in selection medium, the cells were grown to a 'medium' density, harvested and washed 3 times to ensure all traces of geneticin were removed. The cells were then returned to culture for 3-4 days. The supernatant from the cell line was harvested by pelleting the cells by centrifugation (1200 rpm for 5 mins at 4 ^{o}C). The cells were resuspended in cRPMI and cultured for a further 8 passages before being frozen (see section 2.2.3). Supernatant collected at each passage was pooled

and stored at –20°C until GM-CSF was quantified by ELISA (section 2.2.29) and aliquoted into smaller volumes for DC culture (section 2.2.22).

2.2.3 Cryopreservation of cells in liquid nitrogen

Cells were harvested in the same manner as for subculturing and their cell viability assessed. Cells were then centrifuged (1200 rpm for 5 mins) and the pellet resuspended in ice-cold medium containing 10% (v/v) dimethylsulphoxide (DMSO) and 20% (v/v) FCS to give a final cell concentration of 2-4 x 10^6 cells/ml. The cells were then transferred in 1-1.5 ml volumes to cryotubes and frozen in a freezing plug over a liquid nitrogen (N_2) tank, conditions that permitted a gradual decrease in temperature (1°C/min), before being transferred to liquid N_2. To maximise the recovery of cells from liquid N_2, the cells were thawed quickly in a 37°C water-bath and immediately transferred to ice-cold medium. Cells were washed twice in ice-cold medium to ensure all traces of DMSO were removed from the cell suspension. The final pellet was resuspended in complete medium containing 8% (v/v) FCS and cell viability assessed.

2.2.4 CELL COUNTS

Cell counts were performed by diluting cells (usually 1/10 dilution) with trypan blue (Sigma). A 10 µl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (white) were counted using a light microscope.

2.2.5 Assessment of cell viability

An ethidium bromide/acridine orange (EtBr/AO) staining solution was prepared by dissolving 0.1 mg Acridine orange (Sigma, Poole, UK) and 0.1 mg Ethidium bromide (Sigma) in 100 ml of PBS and stored in the dark at 4°C. To assess cell viability 20 µl of cell suspension was diluted in an appropriate volume of EtBr/AO and one drop was placed on a haemocytometer. The number of viable cells (green) and non-viable cells (orange) were counted using a fluorescent microscope with a combination of UV and visible light.

2.2.6 Bacterial strains

Bordetella pertussis Wellcome 28 phase 1 stocks were provided by Keith Redhead at the National Institute for Biological Standards and Control (NIBSC). This strain of bacterium was used in all experiments, unless otherwise stated. *B. pertussis* 338 were also provided by Keith Redhead at NIBSC.

2.2.7 Bacterial Antigens and Vaccines

Formalin-treated and heat killed *B. pertussis* were used in T-cell assays where mentioned. Heat-killed *B. pertussis* were prepared by incubation of cells at 90°C for 20 min. Purified native pertussis toxin (PT), filamentous haemagglutinin (FHA), for use in T-cell assays were provided as gifts from various manufacturers. PT was inactivated by heating at 80°C for 20 min. This abolished mitogenic activity prior to use in T-cell assays *in vitro*. Tracheal cytotoxin (TCT) was provided as a gift from

Prof. William Goldman, Washington University, USA. All other antigens were used as native preparations without chemical or heat inactivation. Freeze dried preparations of acellular pertussis vaccine (ref. no. JHIH-3) and whole cell pertussis vaccine (ref. no. 66-303) were obtained from NIBSC, UK, and were reconstituted in sterile PBS to the appropriate concentration.

2.7.8 UV-STERILISATION

Protein antigens or other preparations were sterilised by exposure to UV-irradiation at 254 nm for 15 mins at a distance of 8 cm from the light source. Only medium-free preparations were UV-irradiated as the phenol red dye present in RPMI-1640 medium generates toxic free radicals upon exposure to UV light

2.29 IMMUNISATIONS:

2.2.9.1 PA AND PW STUDIES

C3H/HeN and C3H/HeJ mice were immunised intraperitoneally (i.p) with $1/5^{th}$ of the human dose of Pa or Pw in 0.3 ml PBS per mouse. Mice received 2 immunisations (week 0 and 4) and were either challenged with *B. pertussis* by aerosol inoculation or sacrificed 2 weeks after second immunisation. 5 mice were used for each experimental group.

2.2.9.2 TCT STUDIES

BALB/c mice were immunised by subcutaneous (s.c.) injection into the footpad with a total volume of 50 µl or into the flank using a total volume of 50 µl (25 µl each side). Mice were injected with PBS or TCT (10 µg/mouse) 6 h before immunisation with various combinations of antigen (KLH, 20 µg/unit) and adjuvants (LT, 100 ng/unit and Alum). After 7-14 d, antigen-specific immune responses were assessed.

2.2.10 AEROSOL INFECTION OF MICE

Mice were infected with *B. pertussis* using a modified version of the method described by Sato *et al.*, (Sato *et al.*, 1980). *Bordetella pertussis* Wellcome 28 phase 1 was grown at 37°C under agitation conditions in Stainer-Scholte liquid medium (Stainer and Scholte, 1970). Bacteria from a 48 hr culture was diluted 1/10 and cultured for a further 48 hrs. Bacteria was then concentrated by centrifugation to 2 x 10^{10} / ml in physiological saline containing 1% casein. In all experiments mice were challenged by exposure for 15 min to the challenge inoculum using a nebuliser directed into a chamber containing groups of 20 – 30 mice. Five mice from each experimental group were sacrificed four hrs after aerosol challenge and at various other times to assess the number of viable bacteria in the lungs.

2.2.11 E NUMERATION OF VIABLE BACTERIA IN THE LUNGS

Lungs were removed aseptically and homogenised in 1 ml of sterile PBS containing 1% casein on ice. 100 µl of neat or serially diluted homogenate were plated out on Bordet-Gengou plates and incubated at 37°C for 4 days. The numbers of colony forming units (CFU) were calculated. Results are given as the mean number of *B. pertussis* CFU for individual lungs from five mice from each experimental group.

2.2.12 B RONCHEOALVEOLAR LAVAGE

The apparatus used for performing broncheoalveolar lavage was constructed in the following manner: A catheter was made by inserting 21-gauge needles into either end of a tightly fitting nylon tube (internal diameter approximately 1.0 mm). The plastic luer lock was then cut off one end of the needles with a small wire cutter to leave the blunt end for insertion into the reaches. The catheter and two 5 ml syringes were then attached to a three-way valve luer lock. Mice were sacrificed and the skin carefully cut and pulled back from the thoracic area. The rib cage, sternum and tissue overlaying the trachea were then carefully removed. Using a 23-gauge needle, a small hole was made in the trachea mid-way between the lungs and the larynx, taking care not to pierce the trachea on either side or the blood vessels either side of the neck. A cotton ligature was then threaded under the trachea using a curved forceps. One of the 5 ml syringes was then filled with warm RPMI 1640 supplemented with 2% FCS. The syringe was re-attached to the three-way valve and the catheter filled with medium. The catheter was inserted into the trachea and secured into position by

tightly tying the cotton ligature. The medium was gently infused into the lungs until they were fully inflated (approximately 0.5 ml). The three-way valve was turned and the fluid withdrawn into the empty syringe. The procedure was repeated until 5 ml of the medium had been infused and withdrawn. Alveolar lavage fluid was then transferred to a 10 ml tube and placed on ice. BAL fluid was centrifuged at 1200 rpm for 5 min. BAL cells were resuspended in RPMI (8% FCS) and a viable cell count was performed. BAL fluid was stored at $-70^{o}C$ until required.

2.2.13 Detection of Immune Responses

Immunised mice were sacrificed by cervical dislocation, usually one or two weeks after one or two immunisations. Sera was collected from immunised and naïve animals and analysed for antigen-specific total IgG or IgG1 and IgG2a subclasses. For *B. pertussis* studies, lungs were also removed and the concentrations of antigen-specific IgA determined. Whole spleens and local lymph nodes from mice were removed and cell suspensions prepared for the detection of cellular immune responses.

2.2.14 Detection of murine T cell responses *ex vivo*

Single cell suspensions were prepared by passing spleens or lymph nodes through a sterile 70 μm cell strainer (NUNC). Spleen cells were centrifuged at 1200 rpm for 5 mins and red blood cells were lysed by resuspending cells in 1 ml of 0.87% ammonium chloride solution for 2 mins. Cells were washed and resuspended in fresh RPMI-1640 medium supplemented with 8% FCS. Spleen mononuclear cells (2×10^{6})

or lymph node cells (1 x 10^6) were cultured in triplicate wells of 96-well 'U bottomed' microtitre plates at 37°C and 5% CO_2 with various concentrations of antigen, or with the mitogens PMA (25 μg/ml; sigma) and anti-mouse CD3 (1 μg/ml; PharMingen, SanDiego, USA) or medium only as positive and negative controls respectively. Supernatants were removed after 72 hours to test for IL-4, IL-5, IFN-γ and IL-10. Cells were cultured for a further 24 hours and pulsed with 0.5 μCi of [^{3}H]-thymidine (Amersham Pharmacia Biotech) in 25 μl of complete RPMI for the final 6 hours of culture. After 6 hours of incubation, cells were harvested onto glass fibre filters (Wallac) with an automatic cell harvester. The filters were then dried using a microwave and sealed in plastic sample bags 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 of spleen cells or lymph node cells.

2.2.15 Assessment of T cell Cytokine concentrations by ELISA

The concentrations of IFN-γ ,IL-4, IL-5, (BD PharMingen) and IL-10 (R&D) were measured by enzyme linked immunosorbent assay (ELISA) using pairs of antibodies as described in Table 2.1. High binding certified ELISA plates (Greiner Bio-one) were coated with 50 μl/well of purified rat anti-mouse IL-5, IL-4 and IFN-γ (1 μg/ml) and IL-10 (2 μg/ml) and plates were incubated overnight at 4°C. The plates were washed 5 times with wash buffer (PBS/0.05% Tween 20) and then incubated with 200 μl/well of blocking solution (PBS supplemented with 10% w/v milk powder for IFN-γ, IL-4 and IL-5 or 5% sucrose/1% BSA w/v solution for IL-10) at room

temperature for 2 hours to prevent non-specific binding. After washing, plates were incubated overnight at 4°C with 50 µl/well of test supernatant or a serially diluted recombinant cytokine standard (Table 2.2). Test supernatant for IL-10 assay was diluted 1:2 with assay reagent diluent (1% BSA solution) according to manufacturers instructions (R&D). Plates were then washed and incubated for either 2 hours at room temperature with 50 µl/well of biotinylated goat anti-mouse IL-10 Ab at 400 ng/ml or 1 hour at room temperature with 50 µl/well of biotinylated anti-mouse IL-4, IL-5 or IFN-γ at 1 µg/ml. Cytokine concentrations in test supernatants and diluted standard concentrations were detected by incubating plates for 30 mins at room temperature with 50 µl/well of HRP conjugated-StrepAvidin (PharMingen, 1:1000 in PBS or R&D, 1:200 in 1% BSA) followed by 50 µl/well of o-phenylenediamine (OPD) substrate (Sigma) dissolved in phosphate citrate buffer, pH 5.0 (0.4 mg/ml). The reaction was allowed to develop for 10 mins, after which, 25 µl/well of 1M H_2SO_4 was added to stop the reaction. Absorbance was read at 492 nm using a microtiter plate reader (Molecular Devices, VERSAmax tunable microplate reader). Concentrations of cytokines in the test supernatants were determined with reference to a standard curve prepared for recombinant cytokines of known concentration. The limits of the assay were 0.5 ng/ml for IFN-γ, 20 pg/ml for IL-4, IL-5 and 16 pg/ml for IL-10.

2.2.16 Determination of serum Total IgG and IgG subclass titres

Serum samples were prepared from peripheral blood removed from the thoracic cavity of mice after sacrifice. Blood was allowed to clot for 2 hrs at room temperature and then placed at 4°C for 2-4 hrs. Serum was transferred into fresh

tubes and stored at -20°C. The levels of antigen specific IgG antibodies in sera of naïve, immunised or infected mice was determined by ELISA. PT, FHA (1 μg/ml in PBS) or *B. pertussis* sonicate (5 μg/ml in PBS) was added to flat-bottomed 96-well microtitre plates and incubated overnight at 4°C. Excess antigen was removed by washing 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 dried milk (Marvel®) for 2 hrs. Sera were serially diluted in PBS on the washed plates to determine the endpoint titres starting at a dilution of 1/50 or 1/100. The plates with serum samples were incubated overnight at 4°C. After washing, plates were incubated for 1hr at room temperature with 50 μl/well of a rat anti-mouse IgG monoclonal antibody (Sigma) (1/1000 dilution in PBS), with specificity for the whole IgG molecule or 50 μl/well of a 1/10,000 dilution of either biotin conjugated IgG2a (0.05 μg/ml) or IgG1 (0.05 μg/ml). Plates were then washed and incubated for 30 min at room temperature with 50 μl/well of HRP-conjugated streptavidin at a 1/1000 dilution in PBS. The plates were then washed and 50 μl of OPD in phosphate citrate buffer (pH 5.0; 0.4 mg/ml) was added to each well. The enzymatic reaction was stopped with 25 μl 1M H_2SO_4. The absorbance was measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres determined by extrapolation of the straight part of the dilution curve to the OD_{492nm} value of the control serum for naïve mice.

2.2.17 IgA ELISA

Lung homogenate samples were prepared from lungs of mice removed from the thoracic cavity after sacrifice. The levels of antigen specific IgA antibodies in the lungs of naïve, immunised or infected mice was determined by ELISA. PT, FHA

(1μg/ml in PBS) or *B. pertussis* sonicate (5 μg/ml in PBS) was added to 96-well microtitre plates and incubated overnight at 4°C. Excess antigen was removed by washing 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 dried milk (Marvel®) for 2 hrs. Lung homogenates were serially diluted in PBS on the washed plates to determine the endpoint titres starting with neat lung homogenates. The plates were incubated overnight at 4°C. After washing, plates were incubated for 1hr at room temperature with 50 μl/well of anti-mouse IgA monoclonal antibody (1/2500 dilution in PBS). Plates were then washed and incubated for 30 min at room temperature with 50 μl/well of HRP-conjugated streptavidin phosphate at a 1/1000 dilution in PBS. The plates were then washed and incubated with 50 μl of OPD in phosphate citrate buffer (0.4 mg/ml). The enzymatic reaction was stopped with 25 μl 1M H_2SO_4. The absorbance was measured at 492 nm. Results are expressed as $\log_{10}$ endpoint antibody titres determined by extrapolation to the OD_{492nm} value of the control naïve lung homogenate.

2.2.18 GENERATION OF *B. PERTUSSIS*-SPECIFIC T CELL LINES AND CLONES

$CD4^+$ T cell lines were generated from the spleens of individual mice 2 weeks after aerosol challenge with *B. pertussis*. Spleen cells were isolated from *B. pertussis* infected C3H/HeN and C3H/HeJ mice were isolated as described in section 2.2.14 and cultured *ex vivo* at $2x10^6$ cells/ml with FHA (5 μg/ml). Recombinant IL-2 (5 IU/ml) was added 5 days later. After a further 7 days, surviving T cells were cultured at $1x10^5$/ml with antigen (FHA, 1 μg/ml; PT, 1μg/ml; formalin treated *B. pertussis* sonicate , 1 μg/ml) and APC (irradiated spleen cells; $2x10^6$/ml), IL-2 was added after

5 days and the stimulate-rest cycle continued every 10-14 d. Cells were tested for antigen-specificity and cytokine production at the end of the rest stage and were cloned by limiting dilution after 2 rounds of antigen stimulation.

2.2.19 Preparation of APC

Whole spleens from naïve C3H/HeN or C3H/HeJ mice were placed in sterile vials containing ice-cold sterile BioWest PBS/8% FCS. These vials were inserted into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30 Gy over 2 mins, which prevents subsequent cell proliferation. The irradiated spleens were homogenised in fresh RPMI-1640 medium supplemented with 8% FCS. The cell suspension was then washed twice, resuspended in fresh medium and cell viability assessed.

2.2.20 Histopathology

Groups of 4 C3H/HeN and C3H/HeJ mice were sacrificed at 7, 14 and 21 days after *B. pertussis* infection. Mice were first anaesthetised with halothane and after initial perfusion with PBS, were perfused via the left ventricle with 10% formol saline for 5 min. The lungs were removed and following overnight fixation in 10% formol saline, dehydrated and embedded in paraffin wax. Sections (4 μM) were cut and routinely stained with haematoxytin and eosin (H&E). Dr. Brian Keogh and Dr. Brian Sheahan assisted with pathology work.

2.2.21 Morphological identif ication of cells in BAL fluid of mice

Bronchoalveolar lavage (BAL) fluids were obtained as described in section 2.2.12. Cells from the lavage fluids were recovered by centrifugation of BAL fluid at 300g for 5 min, and resuspended in RPMI-1640 medium with 8% FCS. 100 µl of the cell suspension was then added into the cytospin wells and centrifuged for 3 mins at 1500 rpm in a cytospin (Shandon). Cells were then fixed by repeated dipping of the slides in 100% methanol for 1 min and then stained for 1 min in eosin red and methyl blue for 1 min. The slide was then carefully washed and allowed to air dry at room temperature. Using a light microscope, a differential cell count was performed on 300 cells/slide and the percentage of macrophages, neutrophils and polymorphonuclear leukocytes was determined. Percentage counts were converted to absolute counts per lung by reference to the total white blood cell count in each BAL sample. Macrophages were identified as large granular cells with abundant cytoplasm, lymphocytes as small sperical cells with little or no visible cytoplasm and neutrophils as small multi-nucleated cells.

2.2.22 G ENERATION OF MURINE BONE MARROW-DERIVED DENDRITIC CELLS

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c ($H\text{-}2^d$), C3H/HeN or C3H/HeJ ($H\text{-}2^k$) mice using a method similar to that described by Lutz *et al.* (Lutz *et al.*, 1999). Mice were euthanized and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with RPMI medium (using a 27 G needle attached to a 10 ml syringe) and cell aggregates were disintegrated (using a 19-G needle attached to a 10 ml syringe).

The resulting cells were washed, pelleted by centrifugation (1200 rpm for 5 mins at 4 °C) and resuspended 2 mls of heated ammonium chloride lysis solution (section 2.1.1) for 2 min to lyse red blood cells. After 2 mins the lysis solution was diluted in the tube by the addition of 25 mls of fresh RPMI medium. The cells were then washed, pelleted by centrifugation (1200 rpm for 5 mins at 4 °C) and resuspended in 5 mls of fresh RPMI medium. Cell viability was assessed by trypan blue method (section 2.2.4) and immature BMDC were prepared by culturing cells at 1 x 10^6 per tissue culture flask in RPMI-1640 and 10% FCS supplemented with 40 ng/ml of GM-CSF in the form of supernatant from a GM-CSF expressing, J558 cell line (provided by Nathalie Winter, Institute Pasteur, Paris, France). After 3 days incubation, 15-20mls of fresh RPMI containing 40 ng/ml GM-CSF (from the J558 cell line supernatant), was added to each culture flask of cells. On day 6, flasks were gently removed from the incubator and cell culture supernatant was carefully removed to eliminate contaminating cells (e.g. granulocytes) from the culture. 10 mls of sterile PBS (BioWest), heated to 37°C, was added to each flask and the flask was gently agitated before the PBS was transferred into 50 ml tubes. 15 mls of sterile EDTA (0.02%; Sigma), heated to 37 °C, was then added to each culture flask and flasks were transferred back into the incubator for 10 minutes. Meanwhile, the cells removed in the PBS step were pelleted by centrifugation (1200 rpm for 5 mins at 4 °C) and resuspended in fresh RPMI medium. After 10 min incubation, culture flasks were removed from the incubator and the EDTA cell solution (removed with vigorous pipetting) was diluted by 15 mls of fresh RPMI medium and cells were pelleting by centrifugation (1200 rpm for 5 mins) and resuspended in 5 mls of fresh RPMI medium. Cells removed by PBS and EDTA steps were pooled and cell viability was assessed by trypan blue method (section 2.2.4). Cells were then recultured at 2 x 10^6

per tissue culture flask in RPMI-1640 and 10% FCS supplemented with 40 ng GM-CSF/ml from the J558 cell line supernatant. After a further 4 days of incubation (day 10), 15-20mls of fresh RPMI containing 40 ng/ml GM-CSF from the J558 cell line supernatant, was added to each culture flask of cells. After a further 2 days in culture (day 12), the loosely adherent cells were harvested, viability of BMDC was assessed and the cells were plated out at required concentration (e.g. 1 x 10^6 cells/ml) in tissue culture plates. DC were plated out overnight in medium containing 10 ng/ml GM-CSF from the J558 supernatant before use.

2.2.23 Generation of murine bone marrow-derived dendritic cells for DC Adoptive transfer *in vivo*

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c mice using the protocol described in section 2.2.22 with the following amendments: The culture medium for DC generation was RPMI supplemented with 2% heat inactivated normal mouse serum. The GM-CSF used was recombinant GM-CSF (BD PharMingen).

2.2.24 Molecules for inducing DC maturation

B. pertussis LPS was supplied by NIBSC. Phosphorothioate-stabilized oligodeoxynucleotide-containing CpG motifs (CpG-ODN) (5'GCTAGACGTTAGCGT) were custom synthesized by Sigma-Genosys. Pam-3Csk4, (palmitoyl-3-cysteine-serine-lysine-4) was purchased from InvivoGen (Autogen Bioclear, UK).

2.2.25 ANALYSIS OF BMDC MATURATION AND CYTOKINE/CHEMOKINE SECRETION

BMDC (10^6/ml) were cultured in 24-well tissue culture plates (Nunc) at 37°C for an appropriate time (as indicated in figure legends). For kinetic studies, DC were cultured in 96-well tissue culture plates and supernatant removed at appropriate time points (2, 4, 6, 12, or 24 h after DC stimulation). In studies examining the immunomodulatory capacities of TCT *in vitro*, DC were cultured at 37°C with TCT (10 μg/ml) for 1, 6 or 12 h before addition of further stimuli (LPS 100- 1000 pg/ml; CpG 100 – 1000 ng/ml and Pam-3Csk 100 –1000 ng/ml) and supernatants removed 12 h later. Supernatants were analysed for IL-12p40, IL-12p70, IL-10, IL-6, TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β) and MIP-2 concentrations by specific ELISA using pairs of antibodies (Table 2.1) as described in the following sections. In some cases, the cells were gently removed from the base of 24-well plates, washed and used for immunofluorescence staining (Section 2.2.31).

2.2.26 IL-12p40 ELISA

IL-12p40 was detected by ELISA, using commercially available antibodies (PharMingen) specific for the IL-12p40 subunit. This antibody recognises IL-12p40 either as a monomer, dimer, or as part of the IL-12p70 or as part of the IL-23 heterodimer. 96-well microtiter plates were coated overnight at 4°C with 50 µl/well of a purified anti-IL-12p40 antibody at a concentration of 2µg/ml. After washing, non-specific binding sites were blocked by adding 200 µl/well of blocking solution (PBS/10% milk powder) and incubating plates at room temperature for 2 hours. Plates were washed and 50 µl/well of test supernatant (diluted 1/500 in RPMI) or serially diluted IL-12 standard (Table 2.2, 0-5000 pg/ml) was added and plates were incubated overnight at 4°C. The plates were then washed and plates were incubated with 50 µl/well of biotinylatyed anti-IL-12p40 (2 µg/ml in PBS) for 1 hour at room temperature followed by 50 µl/well of HRP-conjugated streptavidin (PharMingen, 1:1000 dilution) for 30 mins at room temperature. Finally plates were washed and incubated with 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The enzymatic reaction was stopped with 25 µl 1M H_2SO_4. Absorbance was read at 492 nm and cytokine concentrations in the test supernatants were determined with reference to a standard curve prepared using a recombinant murine IL-12 standard of known concentration.

2.2.27 IL -1β,IL-10, IL-12p70 AND TNF-α ELISA

Cytokine concentrations of IL-1β, IL-10, IL-12p70 and TNF-α were measured using commercially available ELISA kits (R&D systems) (Table 2.1). High-binding

certified 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-1β (4 µg/ml), IL-10 (2 µg/ml in PBS), IL-12p70 (4 µg/ml), TNF-α (0.8 µg/ml) capture antibody in PBS. After washing, non-specific binding sites were blocked by adding 300 µl/well of blocking solution (1% BSA/5% sucrose in PBS) and incubating plates for 2 hours at room temperature. Plates were washed and 50 µl/well of test supernatant (diluted 1:2 in 1% BSA/ PBS) or serially diluted IL-1β standard (0-1000 pg/ml), IL-10 standard (0-2000 pg/ml), IL-12p70 (0-1500 pg/ml), TNF-α(0-2000 pg/ml) in 1% BSA/PBS solution were added and plates were incubated overnight at 4°C. After washing, plates were incubated with 50 µl/well of biotinylated goat anti-mouse biotinylated IL-1β (100 ng/ml), IL-10 (400 ng/ml), IL-12p70 (400 ng/ml), TNF-α (150 ng/ml) in 1% BSA/PBS were added for 2 hours at room temperature. Plates were washed and IL-1β, IL-10, IL-12p70 and TNF-α were detected by incubating plates in the dark for 30 mins with 50 µl/well of horseradish-peroxidase (HRP) conjugated streptavidin (1:200 in 1% BSA/PBS) followed by washing and adding 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The colour reaction was allowed to develop for approximately 10 mins after which time 25 µl/well of 1 M H_2SO_4 was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IL-1β, IL-10, IL-12p70 and TNF-α of known concentration and potency.

2.2.28 IL -6 ELISA

Murine IL-6 was detected by specific immunoassay using matched pairs of monoclonal antibodies (PharMingen, Table 2.1). ELISA plates (high-binding, certified) were coated overnight at 4°C with 50 µl/well of purified rat anti-mouse IL-6 (1 µg/ml in sodium hydrogen carbonate buffer) capture antibody. After washing, non-specific binding was prevented by incubating the plates for 2 hours at room temperature with 200 µl/well of blocking solution (3 % BSA/PBS). The plates were then washed and incubated overnight at 4°C with 50 µl/well of test supernatant or serially diluted IL-6 standard (0-5000 pg/ml in RPMI-1640 medium). After washing, 50 µl/well of biotinylated IL-6 detection antibody (1 µg/ml in PBS) was added and plates were incubated for 1 hour at room temperature. IL-6 was detected by incubating plates for 30 mins at room temperature with 50 µl/well of Streptavidin-HRP (1:1000 in 1 % BSA/PBS) followed by 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). Absorbance was read at 492 nm and concentrations of IL-6 in test supernatants were evaluated from a standard curve prepared using recombinant murine IL-6 of known concentration and potency.

2.2.29 GM -CSF ELISA

GM-CSF concentrations in the J558 cell line supernatant were measured using a commercially available ELISA kit (R & D systems). (Table 2.1). High-binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of rat anti-mouse GM-CSF antibody (2 µg/ml) capture antibody in PBS. After

washing, non-specific binding sites were blocked by adding 300 µl/well of blocking solution (1% BSA/5% sucrose in PBS) and incubating plates for 2 hours at room temperature. Plates were washed and 50 µl/well of test supernatant (diluted 1:2 in 1% BSA/ PBS) or serially diluted GM-CSF standard (0-2000 pg) in 1% BSA/PBS solution were added and plates were incubated overnight at 4°C. After washing, plates were incubated with 50 µl/well of biotinylated goat anti-mouse biotinylated GM-CSF (50 ng/ml), in 1% BSA/PBS were added for 2 hours at room temperature. Plates were washed and GM-CSF was detected by incubating plates in the dark for 30 mins with 50 µl/well of horseradish-peroxidase (HRP) conjugated streptavidin (1:200 in 1% BSA/PBS) followed by washing and adding 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The colour reaction was allowed to develop for approximately 10 mins after which time 25 µl/well of 1 M H_2SO_4 was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse GM-CSF of known concentration and potency.

2.2.30 C HEMOKINE ELISAS

The chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), MIP-2 were detected by ELISA using commercially available antibodies (R&D Systems, Table 2.1). High binding certified ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of MIP-1α (0.4 µg/ml), MIP-1β (2 µg/ml), MIP-2 (2 µg/ml). After washing, plates were incubated for 2 hours at room temperature with 200 µl/well of blocking solution (1% BSA/5% sucrose in PBS), to prevent non-specific binding.

The plates were then washed and 50 µl/well of the test supernatant or serially diluted chemokine standard: MIP-1α (1500 pg/ml), MIP-1β (1500 pg/ml), MIP-2 (1500 pg/ml) in 1% BSA/PBS solution and plates were incubated overnight at 4°C. After washing, the plates were incubated with 100 µl/well of biotinylated detection antibody: MIP-1α (75 ng/ml), MIP-1β (100 ng/ml), MIP-2 (100 ng/ml) for 1 hour at 37°C. The plates were washed and incubated for 30 mins at room temperature with 50 µl/well of Streptavidin-HRP (R & D, 1:200 in /1% BSA/PBS solution). Finally after washing, chemokine concentrations were evaluated by incubating the plates with 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). 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 11.72 pg/ml.

2.2.31 Flow cyto metry analysis

Bone marrow-derived DC surface marker expression was analysed by flow cytometry using a FACS Caliber™ flow cytometer (Becton-Dickinson, San Jose, CA). The FACS was calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads (Calibrate beads, Becton-Dickinson). DC to be analysed were washed in FACS buffer (2% FCS, 0.1% NaN_3 made up in PBS) and transferred to FACS tubes (Falcon). Non-specific binding was prevented by incubating the cells with FACS block (50% FCS: 50% FACS buffer v/v). Cells were then transferred to falcon tubes in 100µl of FACS buffer or 96 well U bottom plates and incubated for 30 minutes with the appropriate FACS antibody and isotype control antibodies. Cells were washed and resuspended in 300µl of FACS buffer and

immunoflourescence analysis was performed. The results were analysed using CELLQuest™ software using a FACS Caliber™ flow cytometer (Becton-Dickson, San Jose, CA). 20,000 cells were analysed per sample, unless otherwise stated.

2.2.32 ADOPTIVE TRANSFER OF DC INTO NAÏVE MICE

BMDC from BALB/c mice were isolated and cultured as described in Section 2.2.22. DC (10^6/ml) were cultured in 24-well tissue culture plates (Nunc) at 37°C for 24 hours with medium alone as a negative control or with KLH (20 μg/ml) alone or with TCT (10 μg/ml) or Pam-3Csk (500 ng/ml) or with TCT (10 μg/ml) and Pam-3Csk (500 ng/ml). After 2 hours the cells were gently removed from the base of the wells and washed five times in RPMI medium to remove all traces of KLH, TCT or Pam-3Csk. Naïve BALB/c mice were injected s.c. (into the flank) with unstimulated or stimulated DC (10^5 cells/mouse) in a total volume of 50 μl. After 14 days, mice were sacrificed and cellular responses were assessed from spleen cells stimulated *ex vivo* with KLH (2, 10, or 50μg/ml) and cytokine concentrations were determined as described in Sections 2.12.1 and 2.12.2.

2.2.33 STATISTICAL ANALYSES

Statistical analyses were performed using the computer-based mathematical package InStat 3. Statistical differences in mean antibody, cytokine or chemokine values between experimental groups were determined by ANOVA. P values of less than 0.05 are considered significant.

TABLE 2.1 ORIGIN AND SPECIFITIES OF ANTIBODIES USED IN ELISAS

ANTIBODY SPECIFICITY	Clone	PURIFIED/CONJUGATE	SUPPLIER
Mouse IFN-γ	RA-6A2	Purified	PharMingen
Mouse IFN-γ	XMG1.2	Biotin	PharMingen
Mouse IL-4	11-B-11	Purified	PharMingen
Mouse IL-4	BVD6-24G2	Biotin	PharMingen
Mouse IL-5	TRFK5	Purified	PharMingen
Mouse IL-5	TRFK4	Biotin	PharMingen
Mouse IL-6	MP5-20F3	Purified	PharMingen
Mouse IL-6	MP5-32C11	Biotin	PharMingen
Mouse IL-12p40	C17.8	Purified	PharMingen
Mouse IL-12p40/70	C17.8	Biotin	PharMingen
Mouse IL-12p70	-	Purified	R & D Systems
Mouse IL-12p70	-	Biotin	R & D Systems
Mouse IL-10	-	Purified	R & D Systems
Mouse IL-10	-	Biotin	R & D Systems
Mouse MIP-1α	-	Purified	R & D Systems
Mouse MIP-1α	-	Biotin	R & D Systems
Mouse MIP-2	-	Purified	R & D Systems
Mouse MIP-2	-	Biotin	R & D Systems
Mouse TNF-α	-	Purified	R & D Systems
Mouse TNF-α	-	Biotin	R & D Systems
Mouse Total IgG	-	Biotin	Sigma
Mouse IgG1	A85-1	Biotin	PharMingen
Mouse IgG2a	R11-89	Biotin	PharMingen
Mouse IgA		Biotin	PharMingen

TABLE 2.2 ORIGIN OF RECOMBINANT CYTOKINES USED AS ELISA STANDARDS

CYTOKINE	SOURCE
Murine IFN-γ	PharMingen
Murine IL-4	PharMingen
Murine IL-5	PharMingen
Murine IL-6	PharMingen
Murine IL-12	PharMingen
Murine IL-12p70	R & D Systems
Murine IL-1β	R & D Systems
Murine IL-10	R & D Systems
Murine MIP-1α	R & D Systems
Murine MIP-2	R & D Systems
Murine TNF-α	R & D Systems

TABLE 2.3 FACS ANTIBODIES AND RELEVANT ISOTYPE CONTROLS

SURFACE MARKER	Stain	ISOTYPE CONTROL	SUPPLIER
CD11c	Tri	Hamster IgG	Caltag
I-A/I-E (MHC class	PE	Rat IgG2a	BD PharMingen
CD40	FITC	Hamster IgG	BD PharMingen
CD86	FITC	Rat IgG2a	BD PharMingen
CD80	PE	Hamster IgG2	BD PharMingen
ICAM-1	FITC	Hamster IgG	BD PharMingen

Chapter 3

The role of TLR4 in shaping the innate and adaptive immune response to Bordetella pertussis

3.1 INTRODUCTION

Pathogen recognition receptors (PRRs), including the Toll-like receptors (TLRs), allow the innate immune system to detect conserved patterns of molecules on pathogens and to respond in the first line of defense against infection by producing inflammatory cytokines (Underhill and Ozinsky, 2002). Furthermore, the innate immune response to pathogens can shape the adaptive immune response, through the stimulation of dendritic cells (DCs), which act as APCs, and also direct the differentiation of naïve T cells. A range of pathogen-derived molecules have been identified as ligands for TLRs. TLR4 was first implicated in LPS recognition (Poltorak *et al.*, 1998), although more recent studies have shown that structurally distinct LPS from certain bacteria, such as, *Leprospira interrogans,* can signal through TLR2 (Pulendran *et al.*, 2001). The role of TLR4 in LPS signalling was identified using LPS-hyporesponsive C3H/HeJ mice, which have a point mutation in the cytoplasmic region of TLR4 resulting in defective TLR4-mediated signalling (Poltorak *et al.*, 1998, Hoshino *et al.*, 1999). Recognition of LPS is initialised by the co-operative interplay between the LPS-binding protein, CD14 and the TLR4-MD2 complex (Akashi *et al.*, 2000). Upon activation by LPS, TLR4 signals via a pathway involving the kinases of the IRAK family, TRAF-6 and NFκB (Zhang *et al.*, 1999).

Binding of pathogens to TLRs on DCs results in their maturation, characterised by upregulation of MHC class II, CD80, CD86, and CD40, but also activation of pro-inflammatory cytokine production, including TNF-α and IL-12. The mature DCs migrate from the tissue to the lymph nodes where they present antigen to naïve T cells. Evidence is emerging that DCs activated by distinct pathogen-derived molecules can selectively promote the induction of distinct T cell subtypes. Many

pathogen-derived molecules, including *E. coli* LPS (Poltorak *et al.*, 1998), unmethylated CpG motifs in bacterial DNA (Hemmi *et al.*, 2000), flagellin (Hayashi *et al.*, 2001) and viral dsRNA (Alexopoulou *et al.*, 2001) that bind TLRs and stimulate IL-12 production by innate cells, such as DCs, direct the induction of Th1 cells. Other pathogen derived molecules, such as yeast hyphae (d'Ostiani *et al.*, 2000), helminth components (Whelan *et al.*, 2000), cholera toxin (Gagliardi *et al.*, 2000) and *Porphyromonas gingivalis* LPS (Pulendran *et al.*, 2001), can activate DCs that drive the differentiation of naïve T cells to a Th2 phenotype. Finally, it has been reported that filamentous haemagglutinin (FHA) and adenylate cyclase toxin (CyaA) from *Bordetella pertussis* can activate innate IL-10 production and stimulate DCs that selectively activate Tr1 cells (McGuirk *et al.*, 2002; Ross *et al.*, 2003).

B. pertussis is a gram-negative bacterium that causes whooping cough, a protracted respiratory disease in young children. Recovery from infection in both children and mice is associated with the induction of *B. pertussis*-specific Th1 cells (Mills *et al.*, 1993; Ryan *et al.*, 1997). Adoptive transfer of Th1 cells from convalescent mice can confer protection (Mills *et al.*, 1993) and IFN-γ receptor knockout mice develop lethal disseminating infection (Mahon *et al.*, 1997). Athymic or SCID mice fail to clear the infection, but the bacteria do not disseminate from the lung (Mills *et al.*, 1993; Harvill *et al.*, 1999), suggesting that innate responses may prevent bacterial dissemination prior to the development of adaptive immunity. Antigen-specific IgG and Th1 responses are suppressed during the acute stages of infection (McGuirk *et al.*, 1998) and it has recently been shown that antigen-specific Tr1 clones can be generated from the respiratory tract of *B. pertussis* infected mice (McGuirk *et al.*, 2002). The Tr1 cells were shown to suppress *B. pertussis*-specific Th1 responses *in vitro* and *in vivo*. The authors concluded that induction of Tr1 cells

may represent an evasion strategy by the pathogen to subvert protective Th1 responses, but also speculated that they may have a role in limiting immunopathology in the lungs (McGuirk *et al.*, 2002).

This present study set out to examine the role of TLR4 in the pathogenesis of *B. pertussis* infection, specifically the role of TLR4 in innate and consequently adaptive immunity to this respiratory pathogen. The findings demonstrate that live or killed *B. pertussis* or *B. pertussis* LPS stimulate DC to produce IL-10, in addition to pro-inflammatory cytokine production and to undergo maturation, through TLR4. *B. pertussis* infection was more severe in TLR4-defective mice, despite an enhancement in antibody and antigen-specific IFN-γ production. However, antigen-specific IL-10 production by T cells was significantly reduced and inflammatory pathology enhanced in TLR4-defective mice, suggesting that TLR4-mediated IL-10 production promotes the generation of Tr1 cells and confers host resistance to the infection by limiting inflammation and collateral damage in the lungs.

Immunological memory is the hallmark of the immune system (Zinkernagel *et al.*, 1996). The characteristic attribute of a memory immune response compared to a primary immune response is its ability to more rapidly generate larger numbers of antigen-specific T cells. Thus, memory T cells do not prevent re-infection or recurrence of quiescent disease, but respond more quickly and efficaciously. This increased kinetics of a memory response is the basis for the success of many vaccines (Esser *et al.*, 2002). This study examined the role of TLR4 in the development of immunological memory to *B. pertussis*. The immune response was not compromised in the TLR4-defective mice. This was evident by the rapid clearance of bacteria from the lungs of both the C3H/HeN and C3H/HeJ mice after re-infection with *B. pertussis*.

Therefore, the defect in triggering the innate immune response in the TLR4-defective mice does not compromise their ability to combat a secondary *B. pertussis* infection.

3.2 RESULTS

3.2.1 *B. pertussis* stimulates proinflammatory cytokine and chemokine production from DCs, but also induces IL-10

Bone marrow-derived DC from BALB/c mice were stimulated with a range of concentrations of killed *B. pertussis*, *B. pertussis* sonicated extract or *B. pertussis* LPS (1.0 μg/ml) and cytokine and chemokine production was measured 18 h later. *B. pertussis* and *B. pertussis* LPS activated DCs to secrete high levels of the pro-inflammatory cytokine, TNF-α, inflammatory chemokine, MIP-1α and the anti-inflammatory cytokine, IL-10 (Fig. 3.1A). Stimulation of DCs with as few as 1 bacterium to 10 DCs stimulated TNF-α production. Low concentrations of sonic extract from *B. pertussis* (which had not been heat-inactivated) were more potent than the equivalent dose of heat-inactivated whole *B. pertussis*. At concentrations equivalent to 100-1000 bacteria to 1 DC, the sonic extract induced lower concentrations of cytokines than the heat-killed whole bacteria, possibly due to the release of inhibitory heat-sensitive bacterial molecules. However, the doses of bacteria employed did not affect DC viability as assessed by staining of dead and viable cells with ethidium bromide/acridine orange.

To investigate the kinetics of cytokine production in response to *B. pertussis*, DCs were stimulated with *B. pertussis* sonicate and heat-killed *B. pertussis* at a ratio of 10 bacteria to 1 DC (or equivalent) or LPS (1.0 μg/ml) and supernatants removed at various time points (0.5 to 48 h). TNF-α production occurred very rapidly; peak concentrations of TNF-α were detected at 2 h (Fig. 3.1B). MIP-1α was first detected

at 2 h but did not peak until 4-8 h after stimulation. In contrast, IL-10 induction was much slower, and was only detectable at 8 h with a peak at 24 h (Fig. 3.1B).

3.2.2 *B. pertussis* induces DC pro- and anti-inflammatory cytokine and chemokine production by signalling through TLR4

Cytokine and chemokine production was assessed 18 h after stimulation of DCs from C3H/HeN and TLR4-defective C3H/HeJ mice with heat killed *B. pertussis*, *B. pertussis* sonic extract, live *B. pertussis* (10 bacteria to 1 DC or equivalent). The dose of bacteria employed was established from dose range experiments with DC from C3H/HeN mice and were very similar to those observed for MIP-1α, TNF-α and IL-10 for DC from BALB/c mice (Fig. 3.1A). In addition, IL-12p70 and IL-6 secretion for DC from C3H/HeN mice was optimal at a ratio of 10 bacteria to one DC. Comparable with DCs from BALB/c mice, DCs from C3H/HeN mice produced TNF-α, IL-12p70, IL-6, MIP-1α, MIP-2 and IL-10 in response to live, heat killed or sonicated *B. pertussis* (Fig. 3.2). IL-12p70, MIP-1α (Fig. 3.2), MIP-1β production was almost completely abrogated in the TLR4-defective C3H/HeJ DCs stimulated with *B. pertussis* ($P<0.001$). Similarly, the production of IL-10 was significantly ($P<0.001$) impaired in C3H/HeJ DCs stimulated with live or killed *B. pertussis* or *B. pertussis* sonicate. The TLR4 defect in C3H/HeJ mice did not have as significant an effect on TNF-α, IL-6 or MIP-2 production by DCs in response to *B. pertussis* (Fig. 3.2), suggesting that components of the bacteria that do not signal through TLR-4 may also promote production of these cytokines. Similar concentrations of MIP-2 were produced by DCs from C3H/HeN and C3H/HeJ mice following stimulation with live *B. pertussis*. However, MIP-2 production in response to heat killed *B. pertussis*

was significantly lower in C3H/HeJ DCs compared with C3H/HeN DCs. In order to confirm that the reduction in cytokine production by DC from C3H/HeJ compared with C3H/HeN mice resulted from the TLR4 defect, rather than other genetic differences, cytokine production by C3H/HeN in the presence of anti-TLR4 antibody was examined. Addition of anti-TLR4 to *B. pertussis*-stimulated DC from C3H/HeN DC significantly reduced production of IL-12p40 11.12 ± 0.2 to 6.2±0.3 ng/ml) (Fig. 3.3).

3.2.3 *B. pertussis* LPS signals through TLR4

Since LPS from many, but not all, bacteria have been shown to signal through TLR4 (Akashi *et al.*, 2000; Werts *et al.*, 2001; Zhang *et al.*, 1999), we examined the role of LPS in the inflammatory cytokine and chemokine responses to *B. pertussis*. The production of all pro-inflammatory cytokines and chemokines examined was significantly ($P<0.01$ - $P<0.001$) lower in the TLR4-defective compared with normal DCs in response to *B. pertussis* LPS (1 μg/ml). Furthermore, IL-10 production was almost completely abrogated in DCs (Fig. 3.4) from the C3H/HeJ mice.

3.2.4 *B. pertussis* LPS induces maturation of DCs through TLR4 signalling, but *B. pertussis* bacteria can also induce maturation of DCs independently of TLR4

Since signalling through TLRs also mediates the signalling events that lead to the maturation of iDC as well as induction of cytokine production, the capacity of *B. pertussis* to induce maturation of murine DC was examined. Bone marrow-derived iDC were cultured for 24 h with *B. pertussis* or with LPS and then analysed for surface expression of costimulatory molecules by immunofluorescence analysis with antibodies specific for CD80, CD86 and CD40. Stimulation with live *B. pertussis* (Fig. 3.5) greatly enhanced CD80 and CD40 expression and moderately enhanced CD86 expression on DC from C3H/HeN and C3H/HeJ mice (Fig. 3.5). In contrast, *B. pertussis* LPS failed to upregulate costimulatory molecule expression on DC from TLR4-defective C3H/HeJ mice, but did activate maturation of iDC from C3H/HeN mice; CD80, CD86 and CD40 were all upregulated on iDC from the C3H/HeN mice stimulated with LPS (Fig. 3.5). The findings demonstrate that *B. pertussis* LPS matures DC by mediating signals through TLR4, but that other components of the bacterium also mature DC independently of TLR4.

3.2.5 Protracted infection in TLR4-defective strain following *B. pertussis* respiratory challenge

Clearance of primary infection with *B. pertussis* appears to be mediated by Th1 cells, but there is also evidence of a role for innate immunity early in the infection (Harvill *et al.*, 1999; Mahon *et al.*, 1997). Here, the role of TLR4 in protective immunity to *B. pertussis in vivo* was examined. C3H/HeN and C3H/HeJ

mice were challenged by exposure to an aerosol of *B. pertussis* and the course of infection was monitored by performing colony forming units (CFU) counts for viable bacteria in the lungs of mice at intervals after challenge. After an initial rise in bacterial counts, the C3H/HeN mice began to clear the bacteria from their lungs 7 days after challenge (Fig. 3.6). In contrast, the number of bacteria in the lungs of the TLR4-defective mice continued to increase until day 14; the CFU counts on days 14 and 21 were significantly higher ($P<0.001$) than in the C3H/HeN strain (Fig. 3.6). The more severe course of infection in C3H/HeJ mice suggests that TLR4 plays a protective role in immunity to *B. pertussis*.

3.2.6 Differential inflammatory cytokine responses in lungs of *B. pertussis* infected C3H/HeN and C3H/HeJ mice

Having demonstrated that cytokine production by DC is significantly compromised in TLR4-defective mice, the inflammatory cytokine and chemokine production in the lungs of C3H/HeJ and C3H/HeN mice following challenge with *B. pertussis* was examined. *B. pertussis* infection of C3H/HeN mice was associated with rapid but transient induction of pro-inflammatory cytokines and chemokines in the lungs (Fig.3.7). In contrast, this early pro-inflammatory response was not detected in the C3H/HeJ strain; significantly higher concentrations of TNF-α, MIP-2 ($P<0.001$), IL-1β and MIP-1α ($P<0.05$) were detected in the infected lungs of C3H/HeN compared to C3H/HeJ mice 3 h post challenge. Transient induction of IL-12p70 was detected at day 7 in C3H/HeN mice and levels were significantly ($P<0.001$) higher than in the C3H/HeJ mice. This suggested that the early innate response to *B. pertussis* was compromised in the TLR4-defective mice. However, pro-inflammatory cytokines were significantly elevated much later (14 days post challenge) in the

TLR4-defective strain; IL-1β, IL-6, IL-12 and MIP-1α concentrations were significantly higher in the lungs of C3H/HeJ compared with C3H/HeN mice 14 days after *B. pertussis* challenge. IFN-γ concentrations also increased in the lungs after infection and the levels at day 7 were significantly greater in the C3H/HeN mice. Overall the concentrations of IFN-γ in the lungs were low in both strains; this is consistent with previous studies of suppressed IFN-γ production locally, but not systemically, during *B. pertussis* infection (McGuirk *et al.*, 1998). High endogenous IL-10 production was found in the lungs of C3H/HeN and C3H/HeJ mice and this increased after infection (Fig. 3.7). The concentrations at day 7 were higher, though not significantly, in C3H/HeN than in the C3H/HeJ mice. However, the concentrations of IL-10 declined to very low levels later in infection (Fig. 3.7).

3.2.7 Enhanced inflammatory infiltrate and exacerbated pathology in the lungs of TLR4-defective mice infected with *B. pertussis*

To further investigate the local response to the *B. pertussis* infection in the lungs, lungs and BAL samples from *B. pertussis* infected C3H/HeN and C3H/HeJ mice were examined for histopathological changes and inflammatory infiltration. A transient infiltrate of neutrophils was detected in BAL fluid from C3H/HeN mice soon after infection with *B. pertussis* (Fig. 3.8). However, this early cellular infiltrate was not observed in lungs of C3H/HeJ mice. In contrast, significantly higher numbers of neutrophils and lymphocytes were detected in the BAL fluid of C3H/HeJ mice 14 and/or 21 days after *B. pertussis* challenge (Fig. 3.8). Macrophages were the dominant cell type in the naïve lung and these levels increased immediately after challenge, especially in the C3H/HeJ mice.

3.2.8 Enhanced pathological changes in the lungs of TLR4-defective mice infected with *B. pertussis*

On histopathological examination, the lungs of C3H/HeN mice appeared normal apart from mild lymphoid peribroncheolar cuffing 14 days post infection (Fig. 3.9). In contrast, the lungs of the TLR4-defective strain exhibited exacerbated damage, with severe peri-bronchoalveolar cuffing and hyperplasia. A dense peri-bronchoalveolar leucocytic infiltrate of neutrophils and mononuclear cells was observed in the C3H/HeJ mice 14 days after challenge (Fig. 3.9). The intra-alveolar fibrin deposits observed are also indicative of severe damage in the lungs of these mice. This severe bronchopneumonia was still evident in the TLR4-defective strain 21 days post challenge.

3.2.9 Reduced antigen-specific IL-10 and enhanced IFN-γ production in *B. pertussis* infected C3H/HeJ mice

The development of adaptive immune responses in TLR4-defective mice was examined. It was observed that *B. pertussis*-specific IL-10 production by spleen cells was significantly lower ($P<0.05$- $P<0.001$) in C3H/HeJ compared with C3H/HeN mice at 14-28 days post *B. pertussis* challenge (Fig. 3.10A). In contrast, *B. pertussis*-specific IFN-γ was significantly higher in spleen cells from the C3H/HeJ strain compared to the C3H/HeN strain 14 days after challenge. However, similar concentrations of IFN-γ were observed in both strains 21-28 days post challenge. *B. pertussis*- specific IL-4 could not be detected in spleen cells from C3H/HeJ or C3H/HeN mice in *B. pertussis* infected mice at any time point examined (data not

shown). Since the killed bacteria may have influenced the *in vitro* responses observed, the responses to purified *B. pertussis* antigens, FHA and PT were also examined. Antigen specific IL-10 production was higher, especially in response to PT, in spleen cells from C3H/HeN mice, whereas IFN-γ production was significantly stronger, especially in response to FHA, in spleen cells from C3H/HeJ mice (Fig. 3.10B).

3.2.10 Neutralisation of IL-10 enhances production of antigen-specific IFN-γ in *B. pertussis*-infected C3H/HeN mice

Enhanced antigen specific IFN-γ and defective IL-10 production was observed in *B. pertussis* infected TLR4-defective mice when compared to the C3H/HeN strain 14 days after challenge. To investigate if the absence of IL-10 production could augment IFN-γ production, a neutralising IL-10 antibody was added to spleen cells from *B. pertussis* infected C3H/HeN mice *ex vivo*. Consistent with the experiments described in Fig 3.10A and 3.10B, antigen-specific IFN-γ was significantly lower in C3H/HeN than in C3H/HeJ mice 14 days after *B. pertussis* infection (Fig. 3.11). However, addition of anti-IL-10 significantly augmented IFN-γ production by spleen cells from C3H/HeN mice in response to FHA or killed *B. pertussis*, suggesting that the reduced IFN-γ production by spleen cells from these mice which compared to the TLR4-defective mice was in part associated with IL-10 production.

3.2.11 CD4+ Tr1 cell lines and clones can be established from C3H/HeN and C3H/HeJ mice after challenge with *B. pertussis*.

In order to confirm that the cytokines detected were secreted by T cells, we established $CD4^+$ T cell lines from C3H/HeN and C3H/HeJ mice 14 and 21 days after challenge with *B. pertussis*. The T cell lines established 14 day after infection of C3H/HeN mice had profiles characteristic either of Tr1 cells (high IL-10, intermediate IL-5, low IFN-γ and no IL-4) or Th1 cells (IFN-γ and no IL-4, IL-5 or IL-10), whereas all T cell lines from C3H/HeJ mice secreted only IFN-γ, characteristic of Th1 cells (Fig. 3.12A). A similar pattern was observed with the T cell lines established 21 days after infection, except that IL-10 was detected at low levels in two of the four T cell lines from the C3H/HeJ mice (Fig. 3.12B). A number of these T cell lines were cloned; 4 T cell clones from one of the T cell lines derived from a C3H/HeN mouse 14 days after *B. pertussis* infection was successfully established. Each of these T cell clones secreted IL-10 and IL-5, but not IFN-γ or IL-4, a cytokine profile characteristic of Tr1 cells (Fig. 3.12C). These findings suggest that IL-10 secreting Tr cells are induced during acute infection of normal mice with *B. pertussis*, but that this response is impaired in TLR4-defective mice.

3.2.12 Enhanced anti body responses in C3H/HeJ mice infected with *B. pertussis*

B. pertussis-specific antibodies were analysed in the sera of *B. pertussis* infected C3H/HeN and C3H/HeJ mice. *B. pertussis*-specific IgG2a was detected 14 days after challenge and reached a peak at day 21 in C3H/HeN mice (Fig. 3.13).

IgG1 antibodies were only detectable from day 21 and the titres were up to 10 fold lower than IgG2a. Antibody responses were not compromised in TLR-4 defective mice; higher titres of *B. pertussis*-specific IgG1, IgG2a and IgG2b were detected in the C3H/HeJ strain when compared with the C3H/HeN strain (Fig. 3.13).

3.2.13 Enhanced IgA response in the lungs of *B. pertussis* infected C3H/HeJ mice

IgA is a major effector of the mucosal immune system (Woof and Kerr, 2004). Therefore, the kinetics of IgA production in the lungs of C3H/HeN and C3H/HeJ mice during the course of *B. pertussis* infection was examined. The *B. pertussis*-specific IgA titres appear enhanced in the lungs of C3H/HeN mice when compared to the TLR4-defective mice 14 and 21 days post challenge (Fig. 3.14). In contrast, the *B. pertussis*-specific IgA titre is significantly higher in the TLR4-defective strain 35 days after infection. The point(s) at which the higher IgA antibody titres are observed in each strain appears to correlate with their respective resolution of *B. pertussis* infection suggesting that IgA contributes to bacterial clearance.

3.2.14 Rapid clearance of *B. pertussis* in lungs of C3H/HeN and C3H/HeJ mice following *B. pertussis* respiratory challenge.

Having established that TLR4 plays a critical role in protective immunity to a primary *B. pertussis* challenge *in vivo* (Figure 3.6), this study next examined the role of TLR4 in the memory response to a secondary *B. pertussis* infection. C3H/HeN and C3H/HeJ mice were challenged by exposure to an aerosol of *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. The

course of infection was monitored by counting bacteria in the lungs of mice at intervals after rechallenge. The number of bacteria in the lungs of both C3H/HeN and the TLR4-defective mice dramatically declined on day 3 and the infection was completely cleared 7 days after rechallenge. The similar kinetics in bacterial clearance from the lungs of both strains of mice suggests that the TLR4 defect does not compromise the development of a memory response to *B. pertussis*.

3.2.15 Early inflammatory cytokine and chemokine responses detected in the lungs of C3H/HeN mice are absent in the TLR4-defective mice after *B. pertussis* rechallenge.

The initial experiments in this study demonstrated an absence of early inflammatory cytokine and chemokine production in the lungs of TLR4-defective mice after primary *B. pertussis* challenge (Fig. 3.7). Here, the kinetics of cytokine/chemokine production in the lungs of C3H/HeN and C3H/HeJ mice following secondary infection with *B. pertussis*. Secondary infection with *B. pertussis* in C3H/HeN mice was associated with rapid but transient induction of proinflammatory cytokines and chemokines in the lungs (Figure 3.16). In contrast, this early proinflammatory response was not detected in the C3H/HeJ strain; significantly higher concentrations of TNF-α, IL-6, MIP-1α, and MIP-2 ($P < 0.001$) were detected in the infected lungs of C3H/HeN compared to C3H/HeJ mice 3 h post rechallenge. This suggests that the early innate response to *B. pertussis* was compromised in the TLR4-defective mice upon rechallenge. The absence of this early proinflammatory response in the lungs did not, however, attenuate the ability of C3H/HeJ mice to clear the infection (Figure 3.15). Therefore, upon secondary

exposure to *B. pertussis*, innate immunity is not of central importance in combating infection.

3.2.16 Enhanced Ag-specific IFN-γ production in TLR4-defective mice following *B. pertussis* respiratory challenge.

Since the extent of $CD4^+$ T cell expansion during a primary response might impact the development of a persistent memory population, the antigen-specific T cell memory responses was examined in C3H/HeN and TLR4-defective mice following re-infection with *B. pertussis*. The development of a memory T cell response was observed 7 days post rechallenge. The FHA-specific IFN-γ production by spleen cells was significantly higher 7, 10 ($p > 0.001$) and 14 days ($p > 0.01$) days after re-infection with *B. pertussis* in the C3H/HeJ compared to C3H/HeN strain (Fig. 3.17). FHA-specific IL-10 production was also significantly higher ($p > 0.05$) in the spleens of C3H/HeJ mice 7 days after secondary infection. This contrasts with the defective Ag-specific IL-10 production during the course of primary infection in TLR4-defective mice.

3.2.17 Similar serum antibody titres in C3H/HeN and C3H/HeJ mice after rechallenge

Higher titres of *B. pertussis* IgG isotypes were detected in the sera of C3H/HeJ mice during the course of primary infection with *B. pertussis* (Figure 3.10). Here, the *B. pertussis*-specific antibodies was examined in the sera of C3H/HeN and C3H/HeJ mice following secondary exposure to *B. pertussis* (Fig. 3.18). *B. pertussis*-specific

antibody titres were observed as early as 3 h after rechallenge and the levels of antibody detected in the sera remained elevated at the intervals examined (3h, 7, 10, or 14 days after rechallenge). The early presence of *B. pertussis*-specific antibody suggests that antibody generated in the primary *B. pertussis* infection was still elevated at the time of rechallenge (Fig. 3.13).

3.2.18 Similar lung IgA responses in C3H/HeN and C3H/HeJ mice after *B. pertussis* rechallenge.

Lung IgA antibody titres were significantly elevated in the latter stages of *B. pertussis* infection in TLR4-defective mice (Fig. 3.14). To investigate if IgA played a role in the rapid resolution of the secondary *B. pertussis* infection, *B. pertussis*-specific IgA antibody titres were quantified in the lungs of C3H/HeN and C3H/HeJ mice after re-infection with *B. pertussis* (Fig. 3.19). Although, not significant, the lung IgA titres in TLR4-defective mice appear enhanced at 3h and 3 days post rechallenge. Elevated lung IgA titres are evident in the lungs of infected C3H/HeN mice 7 days, 10 days ($p < 0.001$) and 14 days after secondary exposure to *B. pertussis*. These results suggest that IgA does not play a pivotal role in bacterial clearance in the memory response to *B. pertussis*.

3.3 DISCUSSION

The significant finding of this study is that signalling through TLR4 plays a crucial role in shaping the innate and consequently the adaptive immune response to *B. pertussis*. TLR4-mediated signalling activates IL-10 production from DCs in response to *B. pertussis*, which promotes IL-10 producing T cells and controls inflammatory pathology during acute infection of the lungs. Although IL-12 and other inflammatory cytokines were also induced by *B. pertussis* via TLR4, induction of antibodies and Th1 responses were not compromised in TLR4-defective mice, suggesting that IL-10 had a dominant effect *in vivo* during acute infection.

Defective innate immune responses to bacterial molecules, including LPS, and increased sensitivity to infection have been reported in mice defective in TLRs (Abel *et al.*, 2002; Kurt-Jones *et al.*, 2000; Wang *et al.*, 2002). TLR4 plays a crucial role in activation of DCs and macrophages by LPS from *E. coli* and Salmonella. However, recent studies indicate that structurally distinct LPS from *Leptospira interrogans* (Werts *et al.*, 2001) employ TLR2 for innate cell activation. This study has demonstrated that LPS from *B. pertussis* stimulates pro-inflammatory cytokine and chemokine production from DCs and maturation of DCs through TLR4. While TLR4 appears to be critical for *B. pertussis* LPS signalling in innate cells, the data suggests that other components of the bacteria stimulated DC maturation independently of TLR4. This finding is consistent with a recent report demonstrating that TLR4 is not required for full maturation of DCs by *Salmonella typhimurium* (Rescigno *et al.*, 2002).

The defective innate response to *B. pertussis* in cells from TLR4-defective mice *in vivo* was reflected in an impaired inflammatory cytokine response in the lungs

of C3H/HeJ mice soon after *B. pertussis* infection. The transient peak of IL-1β, TNF-α, MIP-1α and MIP-2 observed in the lungs of C3H/HeN mice within 3 h of *B. pertussis* challenge was not detected in the C3H/HeJ mice, demonstrating that the initial inflammatory response is much reduced in these TLR4-defective mice. These inflammatory mediators play important roles in leukocyte recruitment in the protective cellular response to bacterial infections (McGuirk and Mills, 2000; Mizgerd *et al.*, 2001; Standiford *et al.*, 1996). *B. pertussis* can be taken up by macrophages and neutrophils and it has been firmly established that Th1 cells can mediate protective cellular immunity to *B. pertussis* (Mills *et al.*, 1993; Ryan *et al.*, 1997). Rapid infiltration of neutrophils into the lungs was demonstrated following *B. pertussis* challenge of naïve BALB/c (McGuirk and Mills, 2000) and C3H/HeN mice (present study), but this early cellular influx was not observed in TLR4-defective mice. Surprisingly, a greater initial recruitment of macrophages was observed in the C3H/HeJ mice, despite the lower levels of inflammatory chemokines in these mice early after challenge. It is possible that a mediator of macrophage recruitment is not affected by the TLR4 defect.

Consistent with the defective innate inflammatory cytokine and chemokine production soon after infection, an increased bacterial burden was observed in the TLR4-defective mice. Delayed clearance of respiratory infections such as *H. influenzae, M. tuberculosis* and respiratory syncytial virus have also been demonstrated in the C3H/HeJ mice (Abel *et al.*, 2002; Kurt-Jones *et al.*, 2000; Wang *et al.*, 2002) and in the case of the bacterial infections, this was attributed to defective innate immunity, in particular inflammatory cytokine production and neutrophil or macrophage recruitment (Abel *et al.*, 2002; Wang *et al.*, 2002). The enhanced *B. pertussis* infection in the TLR4-defective mice was associated with reduced early

inflammatory cell infiltrate into the lungs. However, cellular inflammation and IL-1β, IL-6, IFN-γ and MIP-1α, MIP-2 production in the lungs, were enhanced in the later part of the infection and were associated with a higher bacterial load in the TLR-4-defective mice. In addition, serum antibodies and *B. pertussis*-specific IFN-γ production by spleen cells were enhanced in the C3H/HeJ mice at the peak of infection. This may reflect a higher bacterial burden, but this is unlikely as IFN-γ responses and antibody responses are normally suppressed at the acute stage of *B. pertussis* infection (McGuirk *et al.*, 1998; Mills *et al.*, 1993). Alternatively, the enhanced adaptive immune responses in C3H/HeJ mice may reflect the relative lack of regulatory T cells. Addition of anti-IL-10 *in vitro* enhanced IFN-γ production by spleen cells from *B. pertussis* infected C3H/HeN mice. Furthermore, antigen-specific IFN-γ production was significantly higher and lung infection more severe in IL-10-defective ($IL\text{-}10^{-/-}$) mice (McCann and Mills, unpublished observations), providing support for the conclusion that exacerbated infection was related to the reduced innate and antigen-specific IL-10 production and as a consequence enhanced Th1 and inflammatory responses. Although no major differences in IFN-γ or IL-10 was detected in the lung tissue, significantly higher IL-12p70, IL-1β, IL-6 and MIP1α concentrations in the lungs of C3H/HeJ compared with C3H/HeN mice was observed 14 days after challenge. These cytokines and chemokines play a major role in inflammatory responses in the tissues and are enhanced by Th1 cell-derived IFN-γ or inhibited by IL-10. However, T cell cytokine production is more readily detected ex vivo in peripheral lymphoid organs rather than in the lungs (McGuirk *et al.*, 1998).

Pathological examination of the lungs revealed a dense peribronchiolar infiltrate of neutrophils and lymphocytes in C3H/HeJ mice 14 days after challenge with *B. pertussis*. The intra-alveolar fibrin deposits observed are also indicative of

severe damage in the lungs and this severe bronchopneumonia was still evident in the TLR4-defective strain 21 d post infection. In contrast, the C3H/HeN mice only showed mild lymphoid peribronchiolar cuffing on day 14 of infection. The exacerbated inflammation and lung pathology was associated with decreased antigen-specific IL-10 and normal or increased IFN-γ in the TLR4-defective mice. Although a recent report has suggested that TLR4 is required for optimal development of Th2 responses in an airway inflammation model (Dabbagh *et al.*, 2002), much of the reports to date have suggested that binding of pathogen-derived molecules to TLRs, including *E. coli* LPS binding to TLR4 enhances IL-12 production, leading to the selective activation of Th1 cells (Alexopoulou *et al.*, 2001; Hayashi *et al.*, 2001; Hemmi *et al.*, 2000; Underhill and Ozinsky, 2002). Consistent with these reports, we found that *B. pertussis* bacteria or LPS stimulated IL-12 production was reduced in TLR4-defective mice. However, this study has also demonstrated TLR4-mediated IL-10 production by DCs in response to *B. pertussis* LPS. LPS from *P. gingivalis*, which signals through TLR2, has been shown to enhance induction of Th2 cells, although IL-12, but not IL-4 or IL-10, production was stimulated in splenic DCs (Pulendran *et al.*, 2001b).

It has recently been reported that the LcrV virulence factor from *Yersinia* can trigger IL-10 secretion from macrophages through CD14/TLR2 (Sing *et al.*, 2002). TLR2-deficient mice were less susceptible to oral infection with *Yersinia enterocolitica*, suggesting that the bacteria exploit host innate PRRs to evade protective immune responses. It has been suggested that TLR ligands may induce delayed production of IL-10 by macrophages as a negative feedback to control inflammatory responses (Kopp and Medzhitov, 2003). This study is in agreement with this suggestion; *B. pertussis* induced TNF-α and IL-12 secretion by DCs within

2-4 h of stimulation, whereas IL-10 was detectable after 8 h and peaked at 24 h. However, the data also suggests that reduced IL-10 production as result of defective TLR4 signalling has a more profound effect on the inflammatory response at the peak of infection and the bacterial burden than the reduction in TLR4-mediated IL-12. Studies in our lab have shown that the course of infection and adaptive immune responses are similar in IL-12-defective and wild type mice (Brady and Mills, unpublished observations). Studies with anti-IL-10 antibodies suggested that IL-12 production by innate cells *in vitro* is tightly regulated by IL-10 (McGuirk and Mills, 2000).

It has previously been demonstrated that FHA and CyaA from *B. pertussis* stimulates IL-10 production by DCs and macrophages and promotes the development of Tr1 cells (McGuirk *et al.*, 2002; Mills and McGuirk, 2004; Ross *et al.*, 2004). FHA was found to also enhance LPS-activated IL-10 from macrophages and DCs (McGuirk and Mills, unpublished observations). Thus, it is reasonable to propose that in normal C3H/HeN or BALB/c mice, LPS signalling through TLR4, in cooperation with FHA or CyaA, drives the production of IL-10, which transiently inhibits IL-12 and Th1 responses and promotes the development of Tr1 cells. High levels of *B. pertussis*-specific IL-10 and modest levels of IFN-γ, but undetectable IL-4 was observed in C3H/HeN mice 14-21 days after challenge with *B. pertussis*. In contrast, antigen-specific IL-10 was significantly lower and IFN-γ higher in TLR4-defective mice at the peak of infection. The cytokine profile of antigen-specific T cell lines confirmed the presence of both Th1 and Tr1-type cells in the *B. pertussis* infected C3H/HeN mice but almost exclusively Th1-type in the C3H/HeJ mice. It has previously been reported that antigen-specific Tr1 cells can be isolated from BALB/c mice during acute infection with *B. pertussis* and that these cells can suppress Th1

responses (McGuirk *et al.*, 2002). Furthermore, antigen-specific Tr1 cell lines and clones were generated from mice immunised with antigen in the presence of an adjuvant that enhances LPS-driven IL-10 production (Lavelle *et al.*, 2003). The Tr1-like cell lines had a cytokine profile similar to those generated from C3H/HeN mice in the present study and was also found to suppress IFN-γ production by Th1 cells. The findings of this study suggest that innate IL-10 promotes the activation of IL-10 secreting Tr1 cells during acute infection of normal, but not TLR4-defective, mice and that these cells function to inhibit Th1 responses and limit immunopathology. This is consistent with recent reports demonstrating that $CD4^+$ $CD25^+$ Tr cells may also regulate pathogen-induced inflammation. $CD4^+$ $CD25^+$ T cells from *Helicobacter hepaticus* infected mice block colitis where disease is induced by the transfer of *H. hepaticus*-specific T cells from IL-$10^{-/-}$ mice into in $RAG^{-/-}$ mice (Kullberg *et al.*, 2002). The suppression was mediated by IL-10 and was attributed to *H. hepaticus*-induced Tr cells. Furthermore, while adoptive transfer of $CD4^+CD25^+$ T cells delays bacterial clearance in *Pneumocystis carinii* infected mice, it importantly prevents development of lethal pneumonia (Hori *et al.*, 2002).

Previous studies have indicated that the induction of IL-10 by certain pathogens may represent an evasion strategy to inhibit protective Th1 responses. IL-$10^{-/-}$ mice are more resistant than wild-type mice to *Yersinia* (Sing *et al.*, 2002) and *M. bovis* BCG (Jacobs *et al.*, 2000) infection, through enhanced TNF-α production and cell mediated immunity. However, it has also been demonstrated that the severity of diseases and inflammation, but not the bacterial burden, is exacerbated in IL-10-deficient (IL-$10^{-/-}$) mice infected with *L. monocytogenes, E. coli,* or *H. hepaticus* (Deckert *et al.*, 2001; Kullberg *et al.*, 2001; Sewnath *et al.*, 2001). Increased bacterial load was observed in *B. pertussis* infected IL-$10^{-/-}$ mice, despite the enhanced IgG2a

and Th1 responses at the peak of infection (McCann and Mills, unpublished observations). The findings of this study reveal that IL-10 is produced by cells of the innate immune system through TLR4 and as a consequence Tr cells are activated and this may serve as a protective strategy adopted by the host to limit collateral damage mediated by pathogen-stimulated inflammatory responses.

This study has established that TLR4-mediated signalling plays a critical role in the innate and consequently the adaptive immune response to *B. pertussis*. The intact innate response in the C3H/HeN mice shaped a Th1/Tr1 type response, which led to the resolution of *B. pertussis* infection. In contrast, the attenuated innate response in the TLR4-defective mice led to an exacerbated *B. pertussis* infection as illustrated by the increased bacterial burden and enhanced pathology in the lungs. However, the TLR4-defective mice do, albeit in a delayed fashion, combat *B. pertussis* infection. At present, the mechanism by which the TLR4-defective mice eventually resolve the infection is unclear. The lungs of the *B. pertussis* infected mice exhibited exacerbated damage with severe peri-bronchoalveolar cuffing and hyperplasia 14 days after challenge. This correlates with a peak in the bacterial number in the lungs of the TLR4-defective mice, after which, the infection starts to resolve. Tissue damage induced by infection or injury can result in necrosis, a mode of cell death characterised by the induction of an inflammatory response (Li *et al.*, 2001). A recent study identified necrotic cells as novel and potent inducers of NF-κB. NF-κB activation by necrotic cells resulted in dramatic induction of expression of genes involved in the inflammatory response, such as neutrophil-specific chemokines (e.g. MIP-2) and genes involved in tissue repair. Furthermore, the authors demonstrated that necrotic cell-induced NF-κB was dependent on TLR2 signalling pathways (Li *et al.*, 2001). Importantly, chemokine induction by necrotic cells was

unaffected in macrophages derived from C3H/HeJ mice (Li *et al.*, 2001). A study examining the influence of injury on TLR reactivity using TLR4-mutant C57BL/10ScN/Cr mice revealed that TLR2 responses remained primed, suggesting that injury-induced priming can occur independently of endogenous TLR4 signalling (Paterson *et al.*, 2003). Taken together, the findings of these studies may provide an important insight into how the TLR4-defective mice eventually resolve *B. pertussis* infection. The increased bacterial numbers in the lungs elicit enhanced local damage and necrosis. Necrotic cells in the lung may trigger NF-κB activation via the intact TLR2-mediated signalling pathways in the *B. pertussis* infected C3H/HeJ mice. Importantly, elevated MIP-2 concentrations and neutrophil infiltrate are observed in the lungs of TLR4-defective mice 14 and 21 days after *B. pertussis* challenge. Silverstein (1997), likened pathogen-associated molecular patterns, such as LPS, as "strangers" that activate the innate response (Silverstein and Rose, 1997). In this context, the C3H/HeN mice recognised the "stranger" and mounted an immediate innate response to *B. pertussis*, whilst it appears that it took "danger" in the form of severe injury in the heavily *B. pertussis* infected lungs to enable the TLR4-defective mice to mount a protective immune response.

Memory T-helper cells are critical regulators of long term protective immunity. Two characteristic features of memory cells cause secondary responses to pathogens to be more effective than primary responses. First, the frequency of antigen-specific T- memory cells is much higher than naïve cells (Ahmed and Gray, 1996; Dutton *et al.*, 1998; Sprent and Surh, 2001; Whitmire and Ahmed, 2001). This increase in frequency confers a strong kinetic advantage during the secondary response. This kinetic advantage is evident in the rapid clearance of bacteria from the lungs of both the C3H/HeN and C3H/HeJ mice after re-infection with *B. pertussis*. A

rapid resolution of *B. pertussis* infection was observed in the lungs of TLR4-defective mice despite the absence of early inflammatory cytokines and chemokine production in the lungs after re-infection. This defect in triggering the innate immune response in the TLR4-defective mice does not, however, compromise their ability to combat a secondary *B. pertussis* infection. This study illustrates the importance of this early innate inflammatory response in recruiting macrohages, neutrophils and PMNs to contain the infection during the primary response. The second characteristic of memory T cells is that unlike naïve cells, effector memory T cells make rapid contact with pathogens, through their ability to migrate into inflamed tissue and accumulate at mucosal sites (Hogan *et al.*, 2001b; Hogan *et al.*, 2001a; Masopust *et al.*, 2001; Price and Cerny, 1999; Sallusto *et al.*, 1999; Weninger *et al.*, 2001). This tissue-specific homing ability is linked to the expression of unique combinations of adhesion molecules and chemokine receptors (Austrup *et al.*, 1997; Campbell and Butcher, 2002). The innate inflammatory recruitment signals are therefore redundant in the memory response to *B. pertussis*. Consequently, the TLR4 defect in the C3H/HeJ mice does not compromise their memory response. Furthermore, there is evidence that $CD4^+$ T memory cells that persist in the lung after Sendai virus infection of mice mediate strong protection against secondary infection (Hogan *et al.*, 2001b). Such protection is presumably a reflection of both increased frequency of antigen-specific T cells and the less stringent activation requirements of memory T cells (Sallusto *et al.*, 2004) .

After *in vivo* T cell priming, antigen-specific T cell populations expand, plateau and then contract into long-term memory populations. The size of memory T cell populations correlates with the T cell burst size (Sallusto *et al.*, 2004), suggesting that the processes that drive *in vivo* T cell expansion also influence memory T cell

development (Busch *et al.*, 2000). Consistent with this hypothesis, FHA-specific IFN-γ production by spleen cells was significantly higher in TLR4-defective mice compared to that produced by C3H/HeN strain. Enhanced antigen-specific IFN-γ production by the TLR4-defective strain was also observed in the primary immune response to *B. pertussis*. Surprisingly, the FHA-specific IL-10 response in the TLR4-defective mice was also significantly higher ($p < 0.05$) than that produced by the wild-type strain 7 days after rechallenge. Antigen-specific IL-10 production is however, similar in both strains of mice 10 and 14 days after rechallenge. In this study, defective IL-10 production was observed in the TLR4-defective strain during the primary immune response to *B. pertussis*. A recent study investigated the role of IL-10 in the generation of $CD4^+$ and $CD8^+$ memory T cells showed that IL-$10^{-/-}$ mice had higher frequencies of memory cells (especially $CD4^+$ memory T cells) when compared to the wild-type after second exposure to the antigen (Arps and Kolsch, 2002). The authors concluded that IL-10 may play a role in the generation of memory T cells. This may explain why the recall T cell immune response appears augmented in the C3H/HeJ strain after rechallenge with *B. pertussis*. Central memory T cells are known to be a source of IL-10 (Lanzavecchia and Sallusto, 2000) which may account for the IL-10 production observed in the TLR4-defective strain 7 days after rechallenge.

In conclusion, this study has illustrated a crucial role for TLR4 in the innate and consequently the adaptive immune response to *B. pertussis*. The findings of this study suggest that early TLR4-mediated IL-10 production in response to *B. pertussis* promotes the induction of IL-10-secreting Tr1 cells and consequently inhibits Th1 responses and limits inflammatory pathology in the lungs during infection with *B.*

pertussis. This study has also shown that TLR4-defect in the C3H/HeJ mice does not compromise their ability to combat a secondary *B. pertussis* infection.

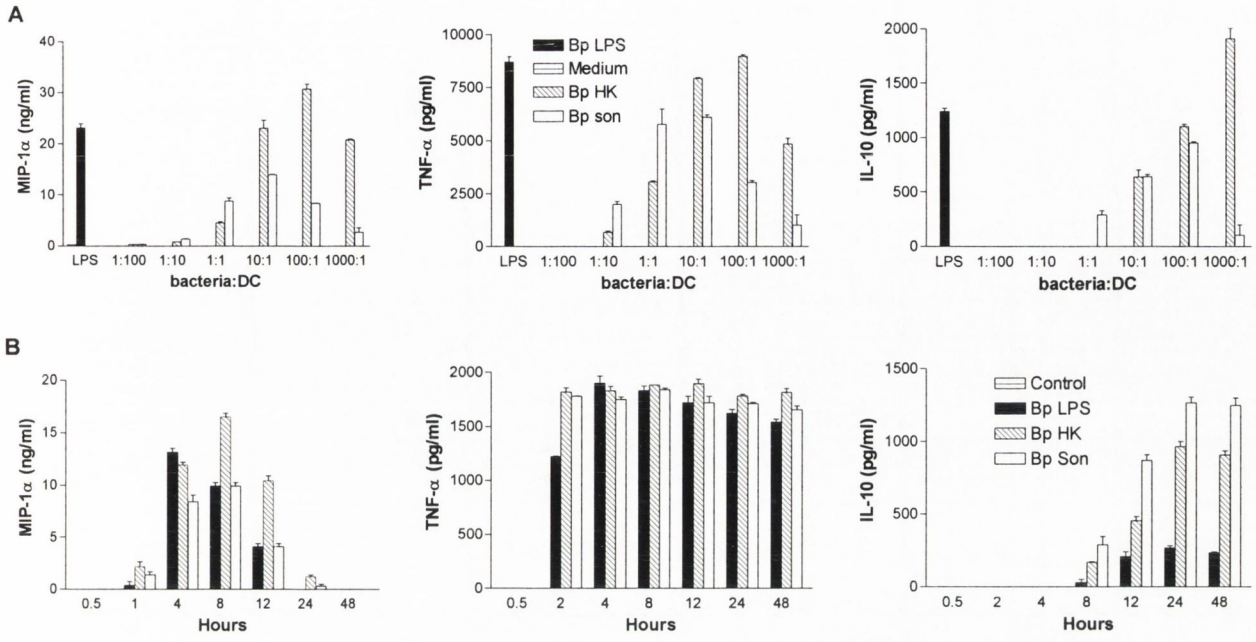


FIGURE 3.1 *B. pertussis* stimulates the production of pro-inflammatory cytokines and chemokines and IL-10 production by DC. (A) Bone marrow-derived DCs from BALB/c mice were stimulated with *B. pertussis* LPS (1 μg/ml), *B. pertussis* sonicate (Bp Son) or heat-killed *B. pertussis* (Bp HK) at ratios equivalent to a multiplicity of infection (M.O.I) of 1 bacterium to 100 DCs to 1000 bacteria to 1 DC. Cytokine and chemokine concentrations were evaluated by immunoassay 18 h later. (B) DCs were stimulated with *B. pertussis* LPS (1 μg/ml), *B. pertussis* sonicate (Bp Son), and heat-killed *B. pertussis* (Bp HK) at 1 DC to 10 bacteria or equivalent and cytokine and chemokine concentrations were evaluated by ELISA 0.5-48 h later. Values represent means ± SD of triplicate assays.

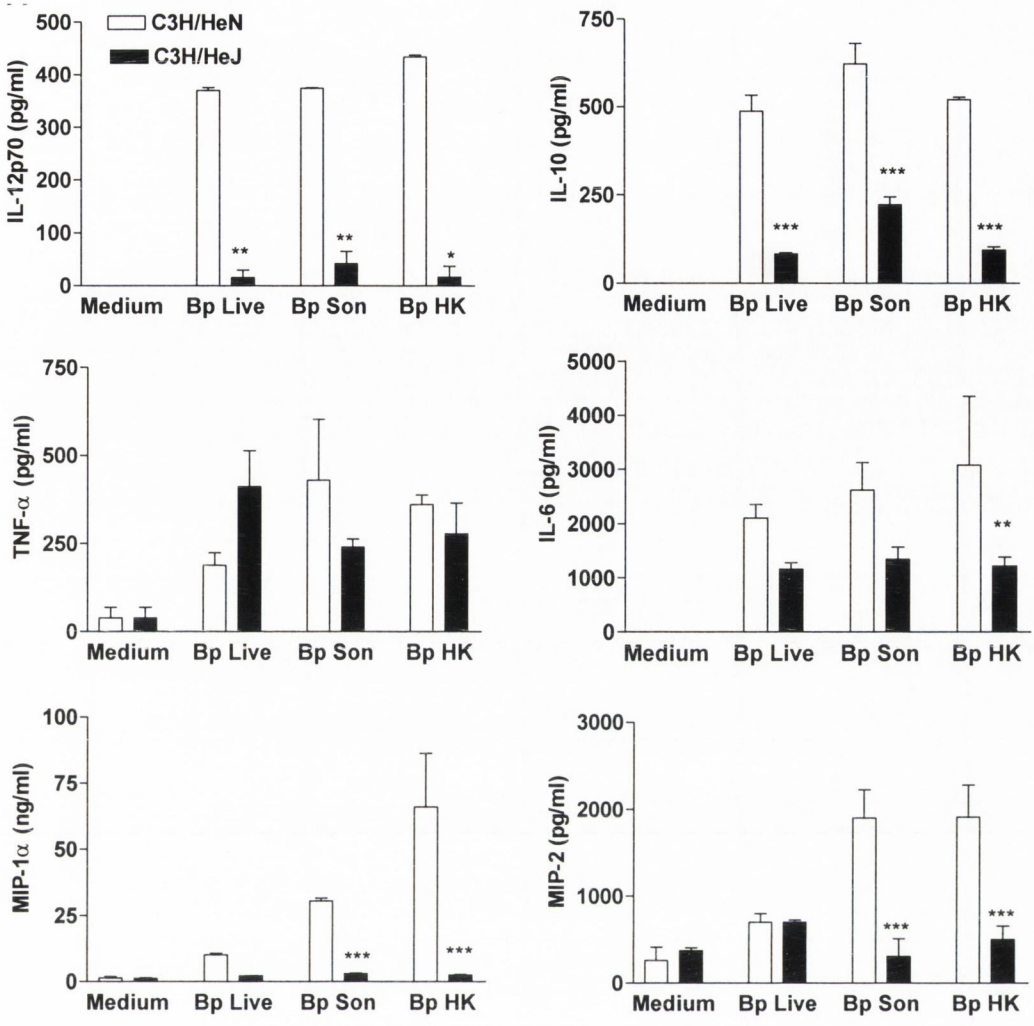


FIGURE 3.2. Impaired *B. pertussis*-induced cytokine and chemokine production by DC from TLR4-defective mice. Bone-marrow-derived DCs from C3H/HeN mice and the TLR4-defective C3H/HeJ mice were stimulated with live *B. pertussis* (Bp Live), *B. pertussis* sonicate (Bp Son) and heat-killed *B. pertussis* (Bp HK) at 10 bacteria to 1 DC (or equivalent). Cytokine and chemokine concentrations were evaluated by ELISA 18 h later. Values represent means ± SD of triplicate assays. $^{**}P<0.01$, $^{***}P< 0.001$ versus C3H/HeN.

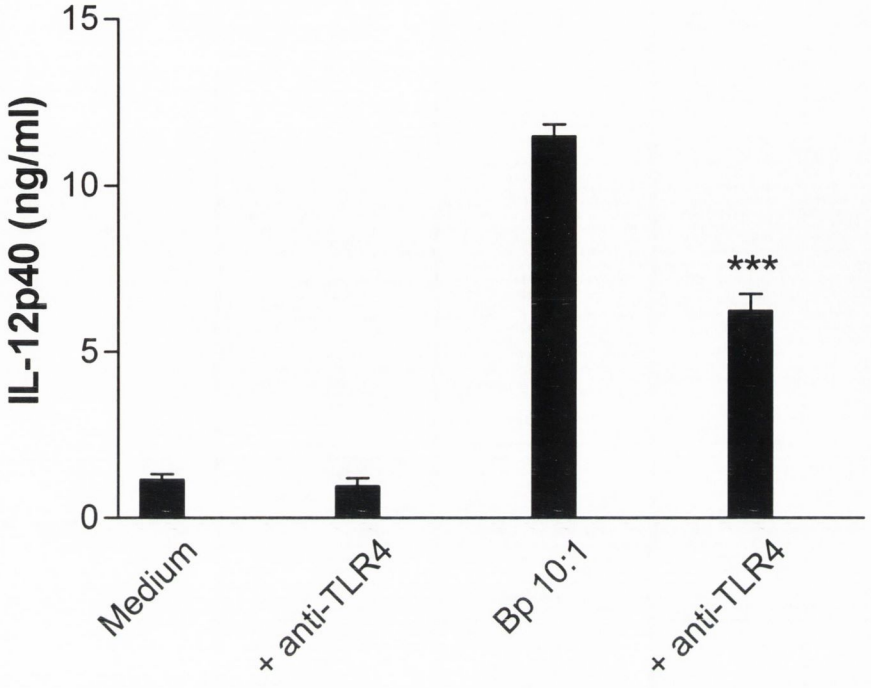


FIGURE 3.3 Bone-marrow-derived DCs from C3H/HeN mice were pretreated with (i) neutralizing TLR4 antibody (10 μg/ml) or (ii) neutralizing IL-10 antibody (10 μg/ml) for 1 h and then stimulated with Bp Live at M.O.I of 10 bacteria to 1 DC. Cytokine and chemokine concentrations were evaluated by ELISA 18 h later. Values represent means ± SD of triplicate assays. $^{**}P<0.01$, $^{***}P< 0.001$ versus C3H/HeN.

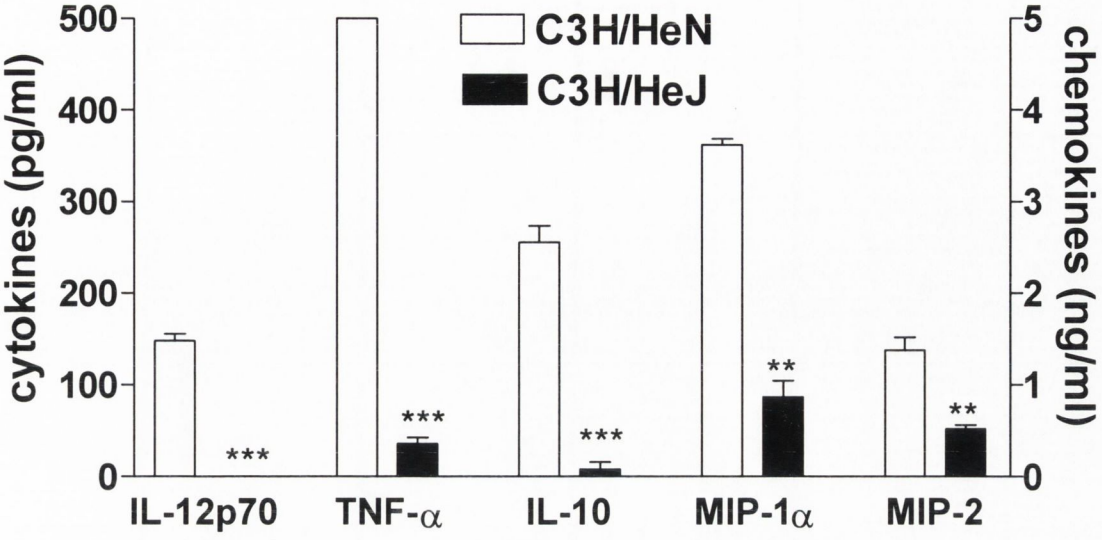


FIGURE 3.4 ***B. pertussis*** **LPS signals through TLR-4.** Bone-marrow-derived DCs from C3H/HeN mice and the TLR4-defective C3H/HeJ mice were stimulated with 1μg/ml *B. pertussis* LPS. Cytokine and chemokine concentrations were evaluated by ELISA 18 h later. Values represent means ± SD of triplicate assays. **P*<0.05, ***P*<0.01, ***P< 0.001 versus C3H/HeN.

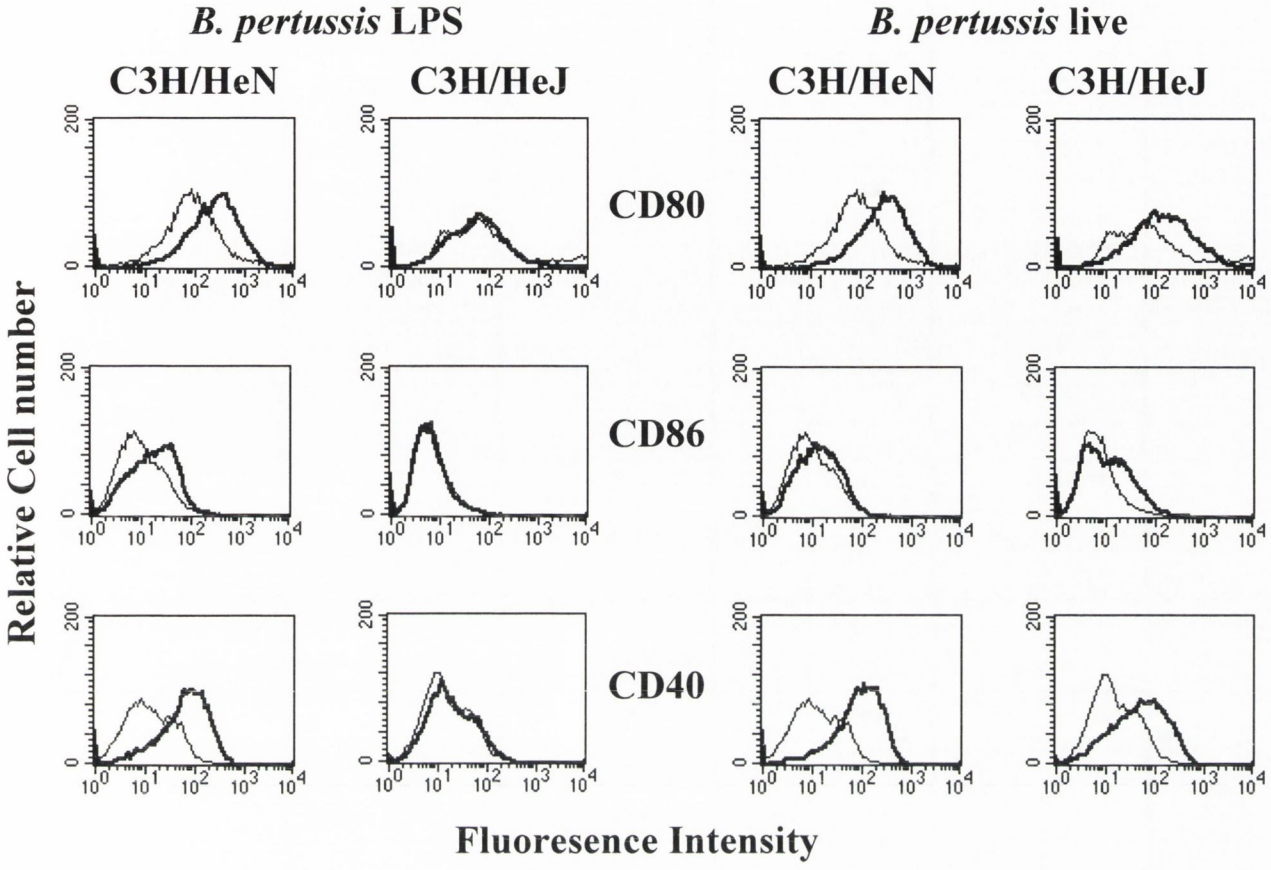


FIGURE 3.5 ***B. pertussis*** **LPS induces maturation of DCs through TLR4, but the whole bacteria induce DC maturation independently of TLR4.** Bone-marrow-derived DCs from C3H/HeN and TLR4-defective C3H/HeJ mice were stimulated with *B. pertussis* LPS (1.0 μg/ml), live *B. pertussis* (10 bacteria: 1 DC) or medium only. After 24 h, cells were washed and immunofluorescence analysis performed with antibodies specific for CD80, CD86 or CD40. Treated DC (bold line) versus untreated DC (thin line). Values represent triplicate wells of triplicate assays

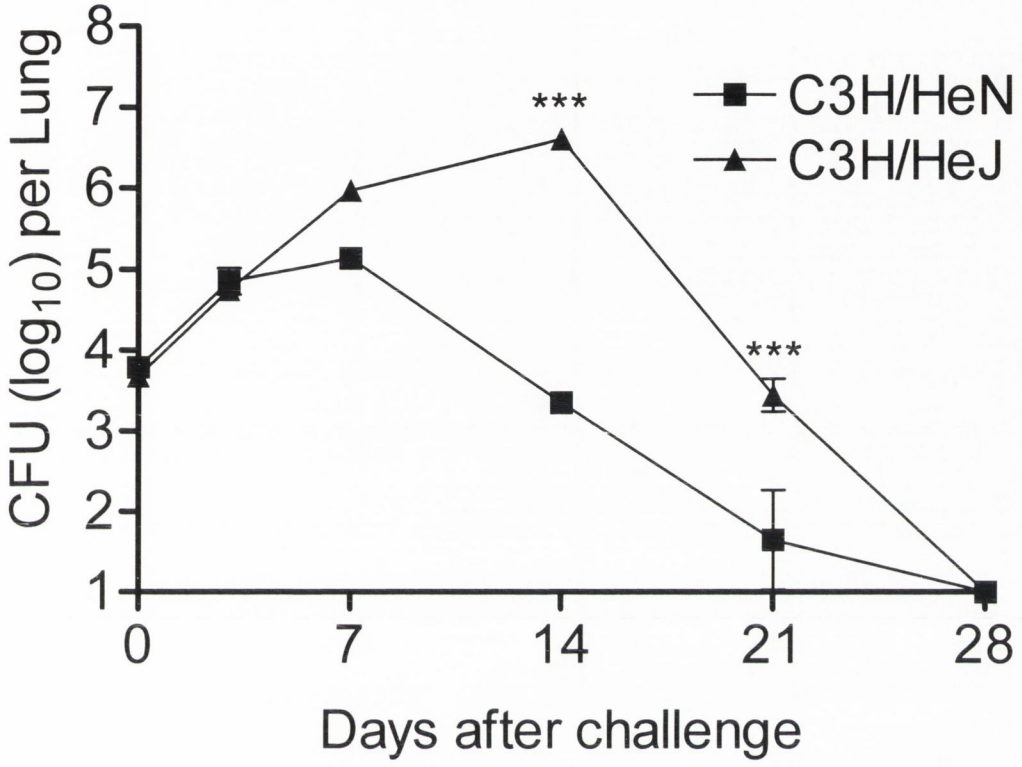


FIGURE 3.6 Increased bacterial burden in the lungs of TLR4-defective mice following *B. pertussis* respiratory challenge. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis* and groups of 4 mice sacrificed 3 h, 3, 7, 14 or 21 days later. Lungs were removed and the number of viable *B. pertussis* determined by performing CFU counts on lung homogenates. ***$P < 0.001$ versus C3H/HeN.

FIGURE 3.7 Differential inflammatory cytokine and chemokine responses in the lungs of C3H/HeN and C3H/HeJ mice infected with *B. pertussis*. Cytokine and chemokine analysis was performed on lung homogenates from C3H/HeN and C3H/HeJ mice 3 h, 7, 14, and 21days after aerosol challenge with *B. pertussis*. Results are mean (± SD) for 4 mice per group at each time point. **P*<0.05, ****P*<0.001 versus C3H/HeN.

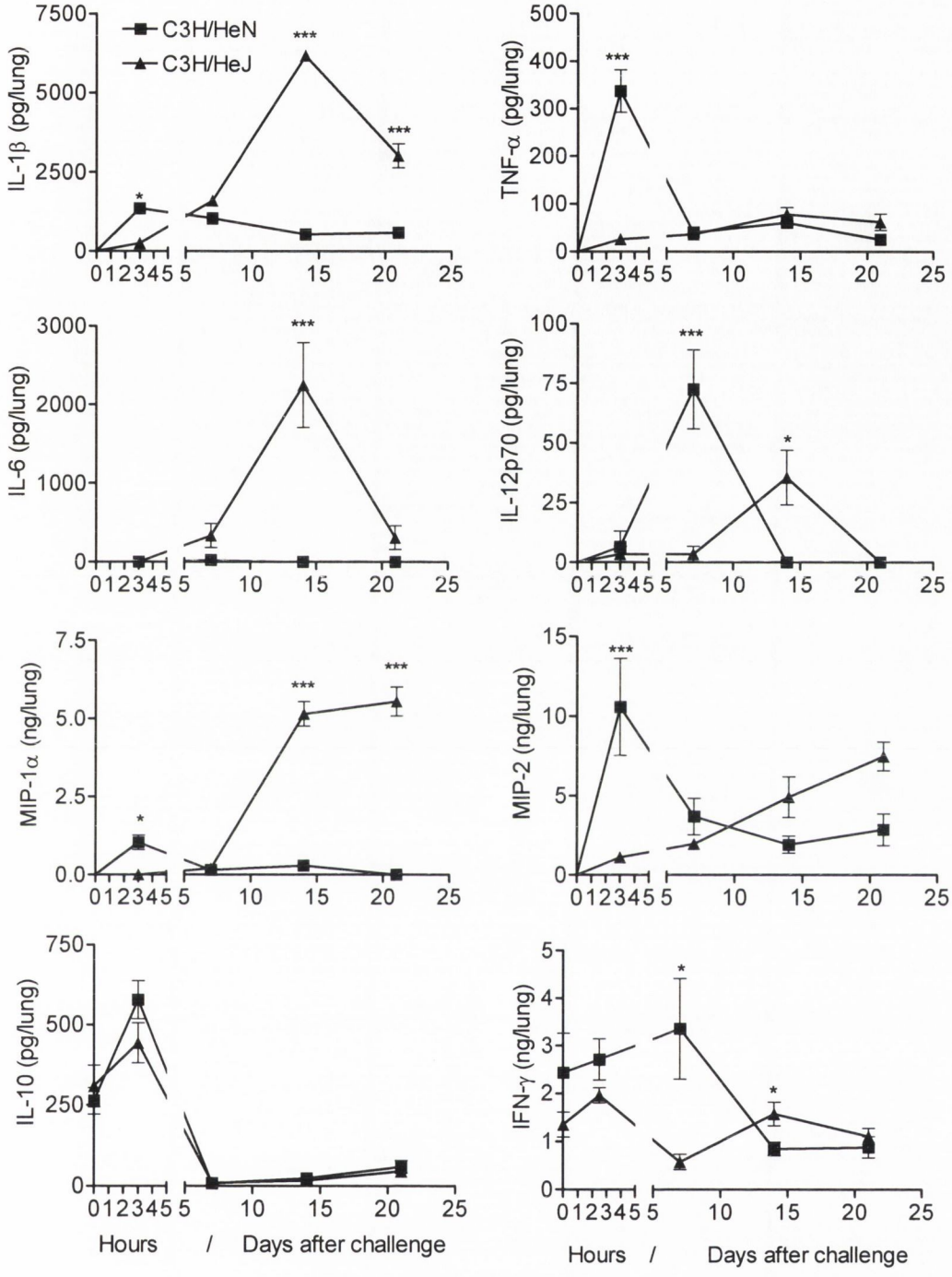

C3H/HeN
C3H/HeJ
IL-1β (pg/lung)
TNF-α (pg/lung)
IL-6 (pg/lung)
IL-12p70 (pg/lung)
MIP-1α (ng/lung)
MIP-2 (ng/lung)
IL-10 (pg/lung)
IFN-γ (ng/lung)
Hours / Days after challenge
Hours / Days after challenge

FIGURE 3.8. Enhanced cellular infiltrate in the lungs of TLR4-defective mice following *B. pertussis* respiratory challenge. BAL fluid was recovered from mice 3 h, 7, 14, and 21 days after *B. pertussis* challenge and assessed for cellular composition by microscopic examination of Diff-Quik stained cytospins. Results are means (±SD) for 4 mice per group at each time point. $^{**}P<0.01$, $^{***}P<0.001$ versus C3H/HeN.

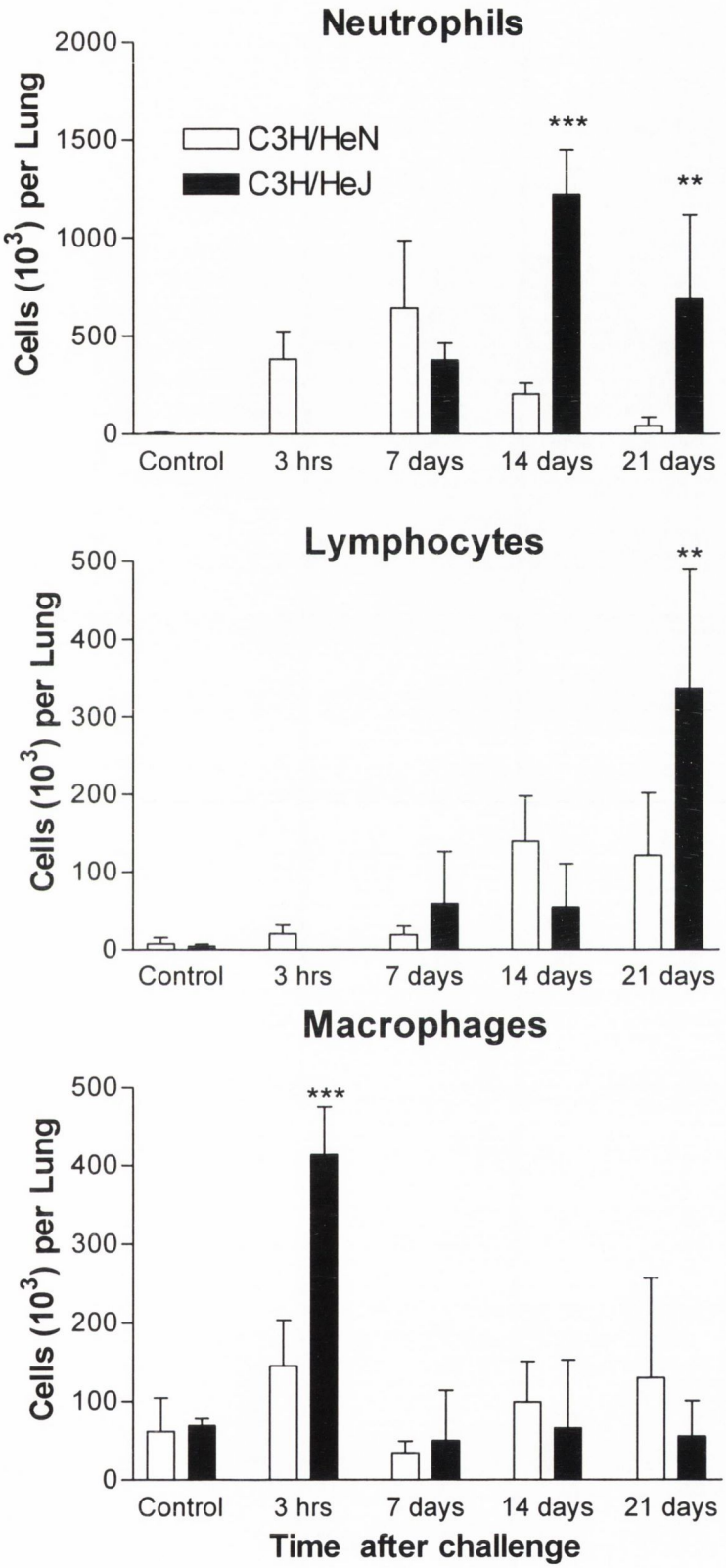

Neutrophils
2000
1500
1000
500
0
Cells (10^3) per Lung
C3H/HeN
C3H/HeJ
***
**
Control
3 hrs
7 days
14 days
21 days
Lymphocytes
500
400
300
200
100
0
Cells (10^3) per Lung
**
Control
3 hrs
7 days
14 days
21 days
Macrophages
500
400
300
200
100
0
Cells (10^3) per Lung
***
Control
3 hrs
7 days
14 days
21 days
Time after challenge

FIGURE 3.9. Enhanced pathological changes in the lungs of TLR4-defective mice infected with *B. pertussis*. (A) C3H/HeN mouse, 14 days post *B. pertussis* infection. Small numbers of lymphocytes in peribronchiolar and perivascular spaces. Alveoli appear normal. (B) C3H/HeN mouse, 21 days post infection. Mild peribronchiolar and perivascular lymphocyte accumulations. (C) C3H/HeJ mouse, 14 days post infection. Perivascular neutrophil accumulations and hyperplasia of bronchiolar epithelium. Alveolar walls are thickened and neutrophils and macrophages are present in alveolar spaces. Alveoli appear normal. (D) C3H/HeJ mouse, 21 days post infection. Intra-alveolar macrophages and neutrophils and thickening of alveolar walls. Mild lymphocyte accumulations in perivascular areas. (H&E, original magnification x200).

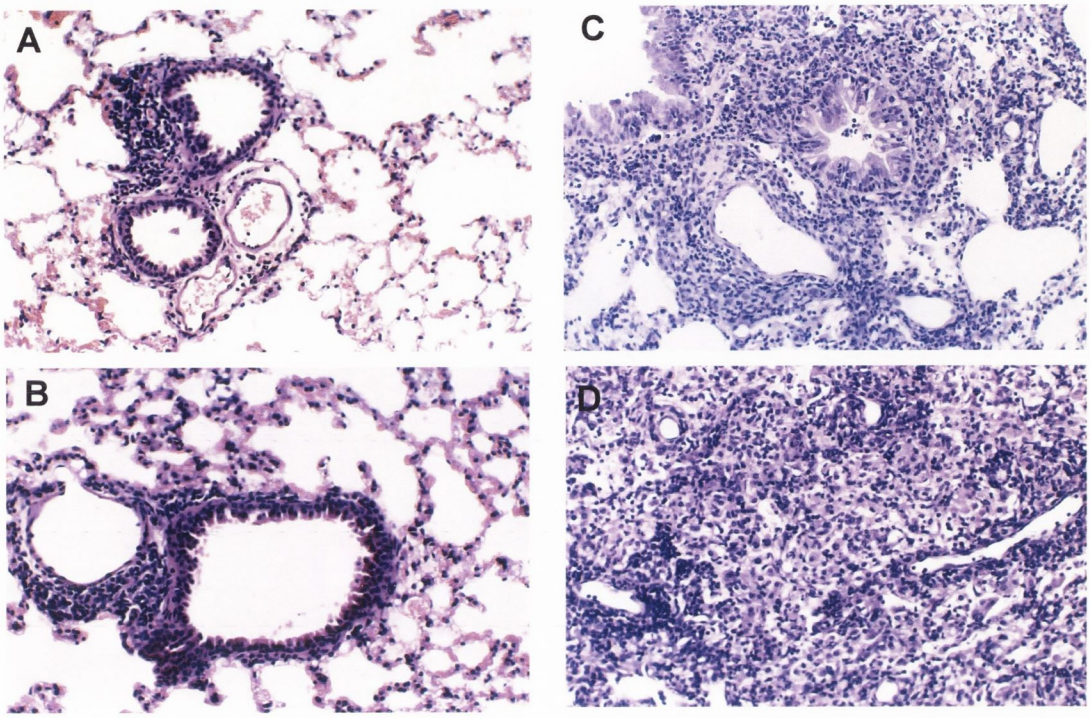
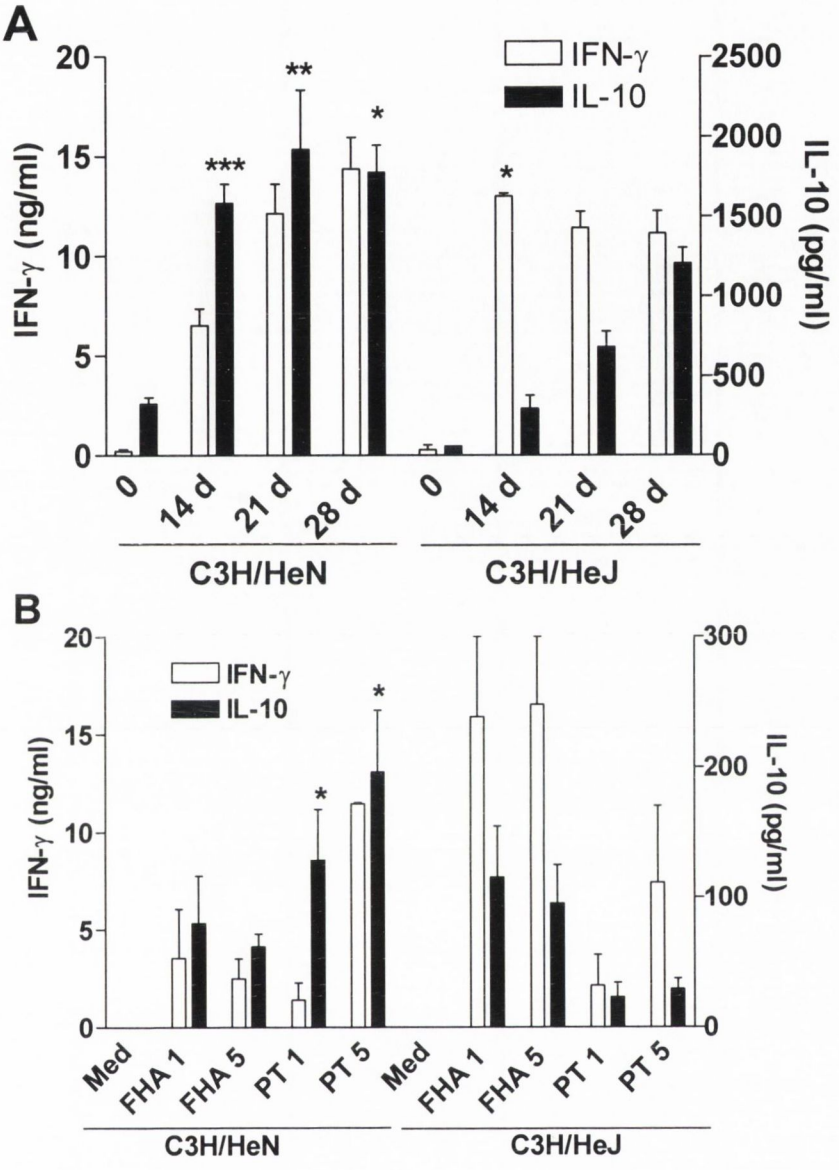

A
C
B
D

FIGURE 3.10. Reduced antigen-specific IL-10 producing T cells in TLR4-defective mice infected with *B. pertussis*. A) Spleen cells prepared from *B. pertussis* infected C3H/HeN and C3H/HeJ mice 0-28 d post challenge with *B. pertussis* were stimulated with antigen (1x 10^5 killed *B. pertussis*/ml). Supernatants were removed after 3 days and tested for IFN-γ and IL-10 by ELISA. Responses of cells to medium only were negative and are not shown. B) Spleen cells from C3H/HeN and C3H/HeJ mice 14 days after challenge with *B. pertussis* were stimulated with FHA or PT (1 or 5 μg/ml) or medium only, supernatants were removed after 3 days and tested for IFN-γ and IL-10. Results are mean (±SD) values for 5 mice per group and are representative of 3 experiments. **P*<0.05, ***P*<0.001, ****P*<0.001 C3H/HeN versus C3H/HeJ.


A
IFN-γ
IL-10
IFN-γ (ng/ml)
IL-10 (pg/ml)
20
15
10
5
0
2500
2000
1500
1000
500
0
***
**
*
*
0
14 d
21 d
28 d
0
14 d
21 d
28 d
C3H/HeN
C3H/HeJ
B
IFN-γ
IL-10
IFN-γ (ng/ml)
IL-10 (pg/ml)
20
15
10
5
0
300
200
100
0
*
*
Med
FHA 1
FHA 5
PT 1
PT 5
Med
FHA 1
FHA 5
PT 1
PT 5
C3H/HeN
C3H/HeJ

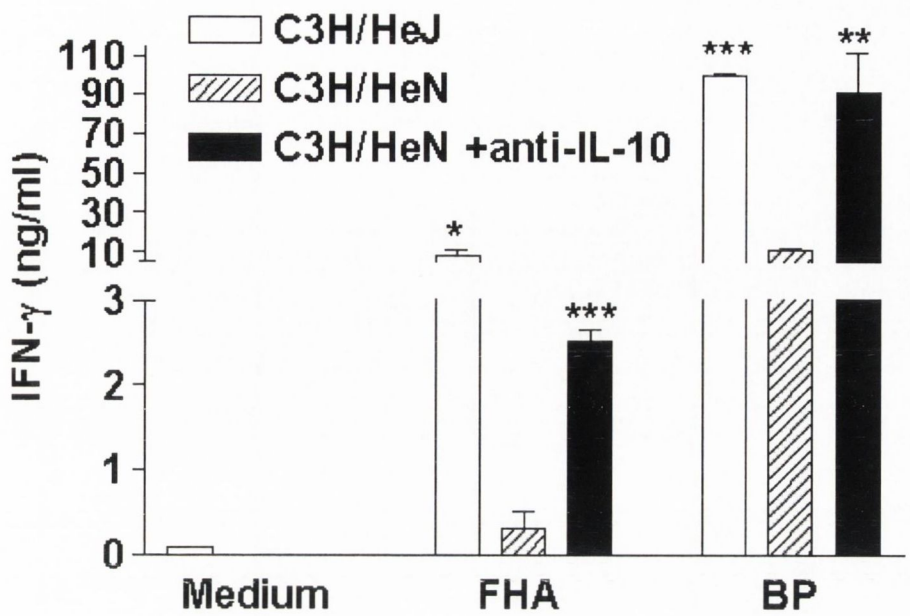
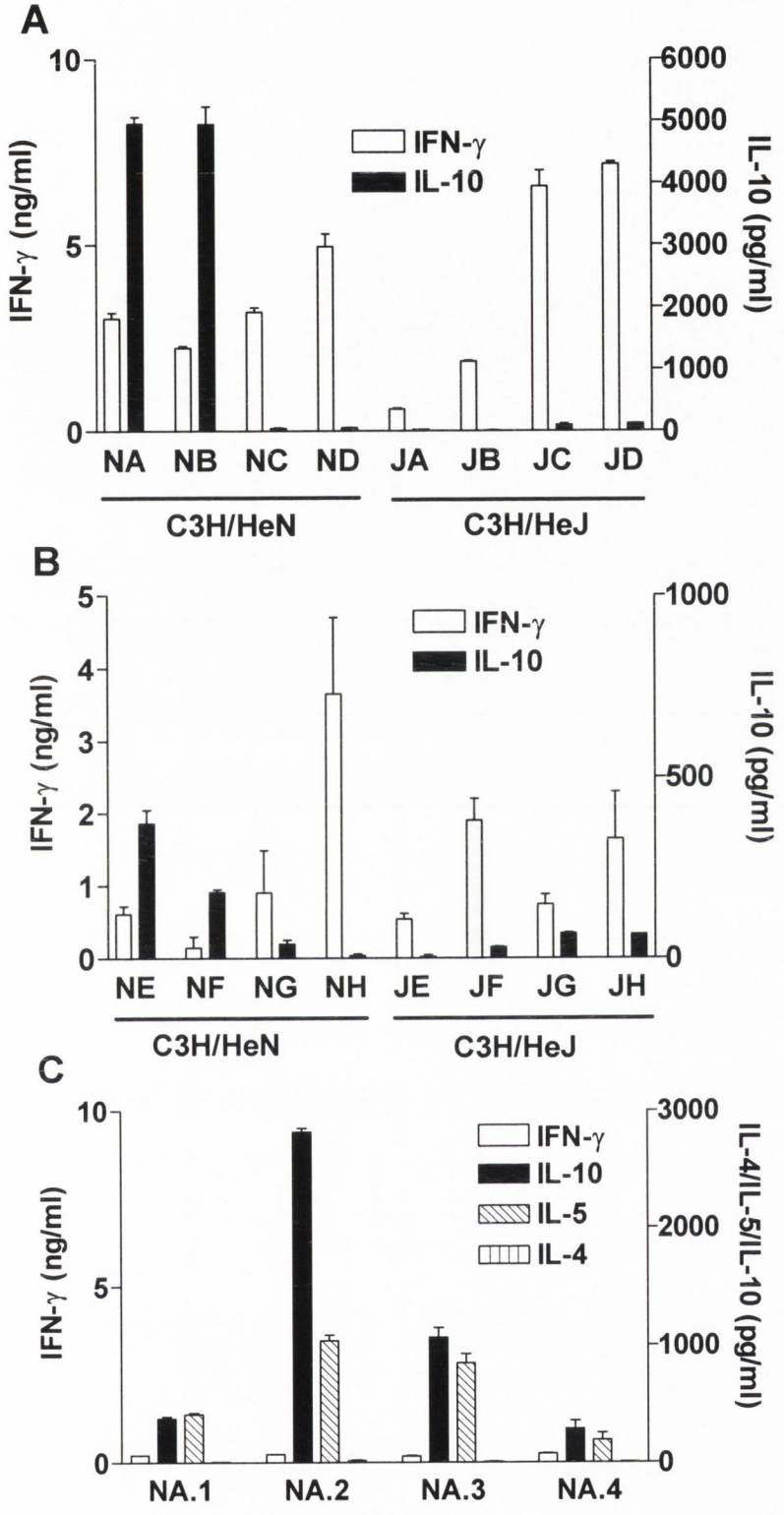


FIGURE 3.11. Enhanced production of antigen-specific IFN-γ in C3H/HeN mice after addition of an anti-IL-10 antibody. Spleen cells from C3H/HeN and C3H/HeJ mice 14 days after challenge with *B. pertussis* were stimulated with FHA (1 μg/ml) or killed *B. pertussis* (BP; 1x 10^5 killed *B. pertussis*/ml) or medium only. Spleen cells from C3H/HeN mice were also cultured with antigens in the presence of anti-IL-10 (10 μg/ml). Supernatants were removed after 3 days and tested for IFN-γ. Results are mean (±SD) values for 4 mice per group and are representative of 3 experiments. **P*<0.05, ***P*<0.001, ****P*<0.001 C3H/HeN versus C3H/HeJ or C3H/HeN + anti-IL-10.

FIGURE 3.12. $CD4^+$ Tr1 cell lines and clones established from C3H/HeN and C3H/HeJ mice. FHA-specific $CD4^+$ T cell lines were established from spleens of individual C3H/HeN and C3H/HeJ mice 14 (A) or 21 (B) days after challenge with *B. pertussis*. C) T cell clones were established from T cell line NA generated from a C3H/HeN mouse 14 days after *B. pertussis* challenge. T cells lines or clones were stimulated with APC + antigen and IFN-γ, IL-4, IL-5 and IL-10 tested in supernatants 3 days later. Results are mean values for triplicate cultures.


A
10
5
0
IFN-γ (ng/ml)
6000
5000
4000
3000
2000
1000
0
IL-10 (pg/ml)
IFN-γ
IL-10
NA NB NC ND JA JB JC JD
C3H/HeN
C3H/HeJ
B
5
4
3
2
1
0
IFN-γ (ng/ml)
1000
500
0
IL-10 (pg/ml)
IFN-γ
IL-10
NE NF NG NH JE JF JG JH
C3H/HeN
C3H/HeJ
C
10
5
0
IFN-γ (ng/ml)
3000
2000
1000
0
IL-4/IL-5/IL-10 (pg/ml)
IFN-γ
IL-10
IL-5
IL-4
NA.1 NA.2 NA.3 NA.4

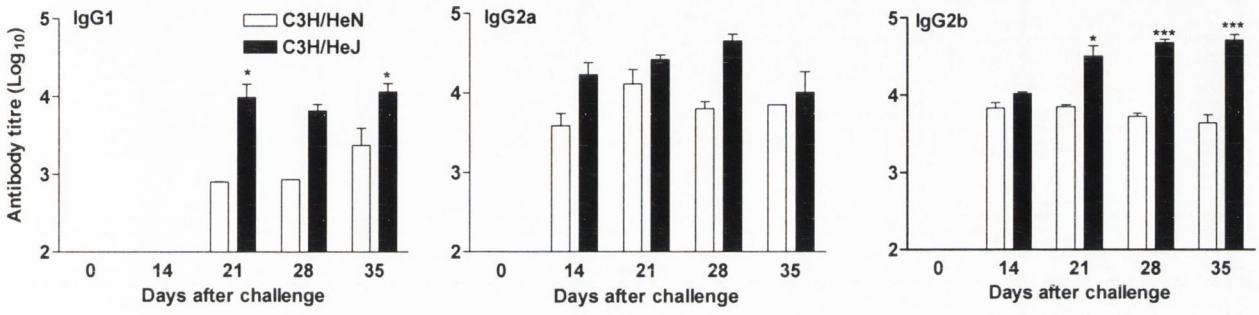


FIGURE 3.13 Enhanced antibody production in TLR4-defective mice after *B. pertussis* infection. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*. Antibody titres to *B. pertussis* (using sonicated *B. pertussis*, 5 μg/ml) in the sera of both strains of mice were analysed by ELISA 14, 21, 28 and 35 days after *B. pertussis* challenge. Results are mean (±SD) values for 4 mice per group. **P*<0.05, ***P*<0.001, ****P*<0.001 C3H/HeN versus C3H/HeJ.

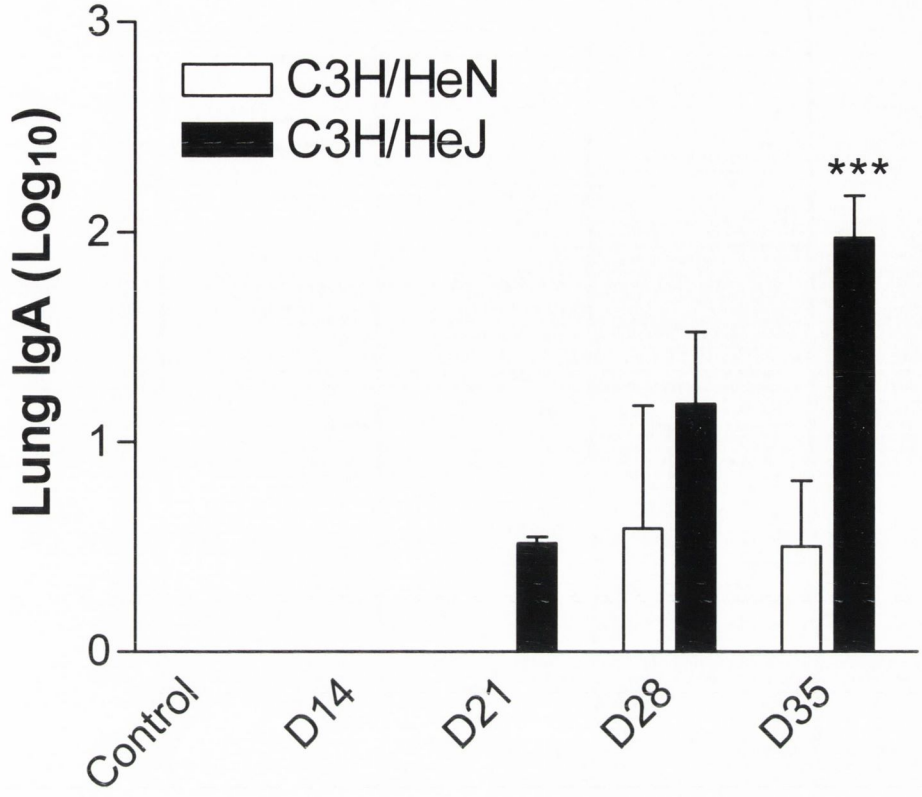


FIGURE 3.14 Enhanced IgA response in the lungs of *B. pertussis* infected C3H/HeJ mice. C3H/HeN and C3H.HeJ mice were aerosol challenged with *B. pertussis*, and groups of four mice were sacrificed 3h, or 3, 7, 14 or 21 days later. Lungs were removed and IgA responses to sonicated *B. pertussis* (5 μg/ml) were evaluated by immunoassay. Results are expressed as mean antibody titres (± SD) for four mice per group. ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

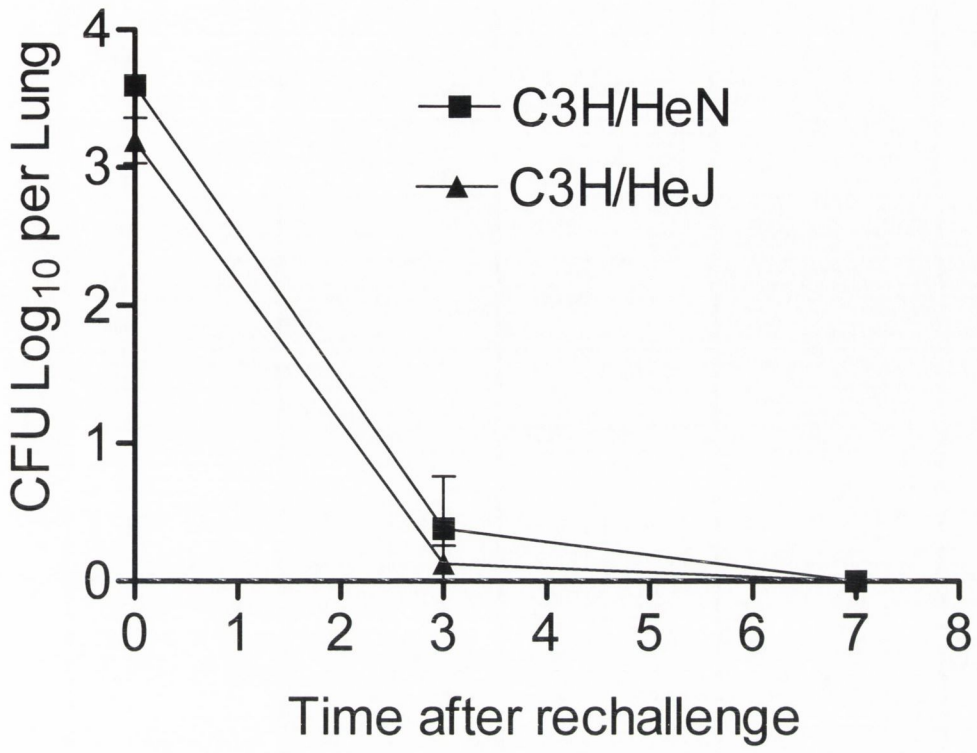


FIGURE 3.15 Rapid clearance of *B. pertussis* in lungs of C3H/HeN and C3H/HeJ mice following *B. pertussis* respiratory rechallenge. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. Groups of four mice were sacrificed 3h, 3, 7, 10, and 14 days after rechallenge. Lungs were aseptically removed and the number of viable *B. pertussis* was determined by performing CFU counts on lung homogenates. Results are mean (± SD) for four mice per group at each time point.

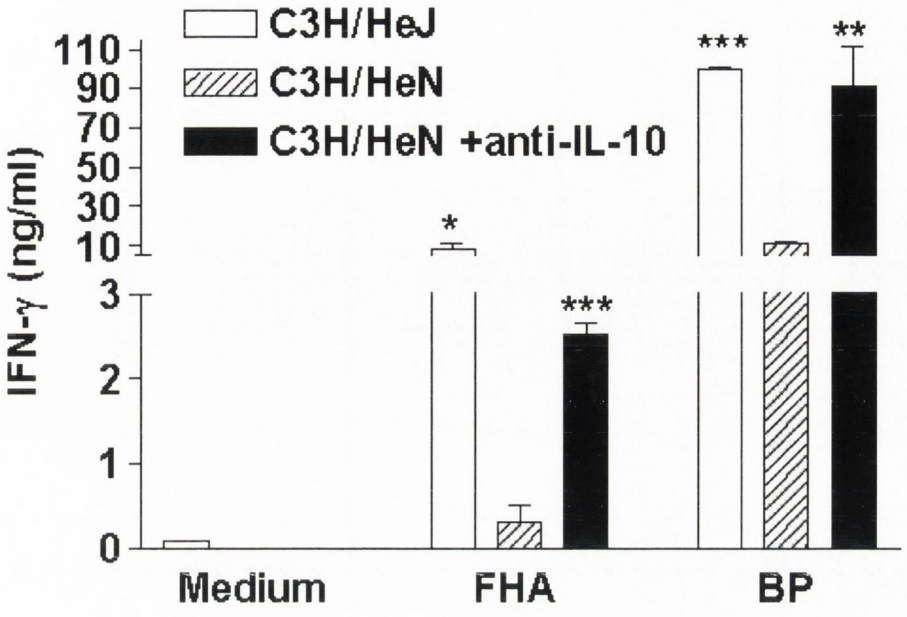


FIGURE 3.11. Enhanced production of antigen-specific IFN-γ in C3H/HeN mice after addition of an anti-IL-10 antibody. Spleen cells from C3H/HeN and C3H/HeJ mice 14 days after challenge with *B. pertussis* were stimulated with FHA (1 μg/ml) or killed *B. pertussis* (BP; 1x 10^5 killed *B. pertussis*/ml) or medium only. Spleen cells from C3H/HeN mice were also cultured with antigens in the presence of anti-IL-10 (10 μg/ml). Supernatants were removed after 3 days and tested for IFN-γ. Results are mean (±SD) values for 4 mice per group and are representative of 3 experiments. **P*<0.05, ***P*<0.001, ****P*<0.001 C3H/HeN versus C3H/HeJ or C3H/HeN + anti-IL-10.

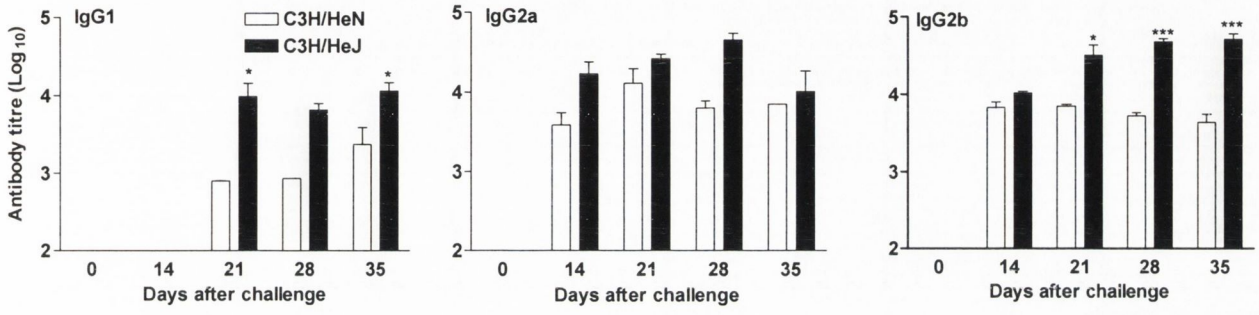


FIGURE 3.13 Enhanced antibody production in TLR4-defective mice after *B. pertussis* infection. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*. Antibody titres to *B. pertussis* (using sonicated *B. pertussis*, 5 μg/ml) in the sera of both strains of mice were analysed by ELISA 14, 21, 28 and 35 days after *B. pertussis* challenge. Results are mean (±SD) values for 4 mice per group. **P*<0.05, ***P*<0.001, ****P*<0.001 C3H/HeN versus C3H/HeJ.

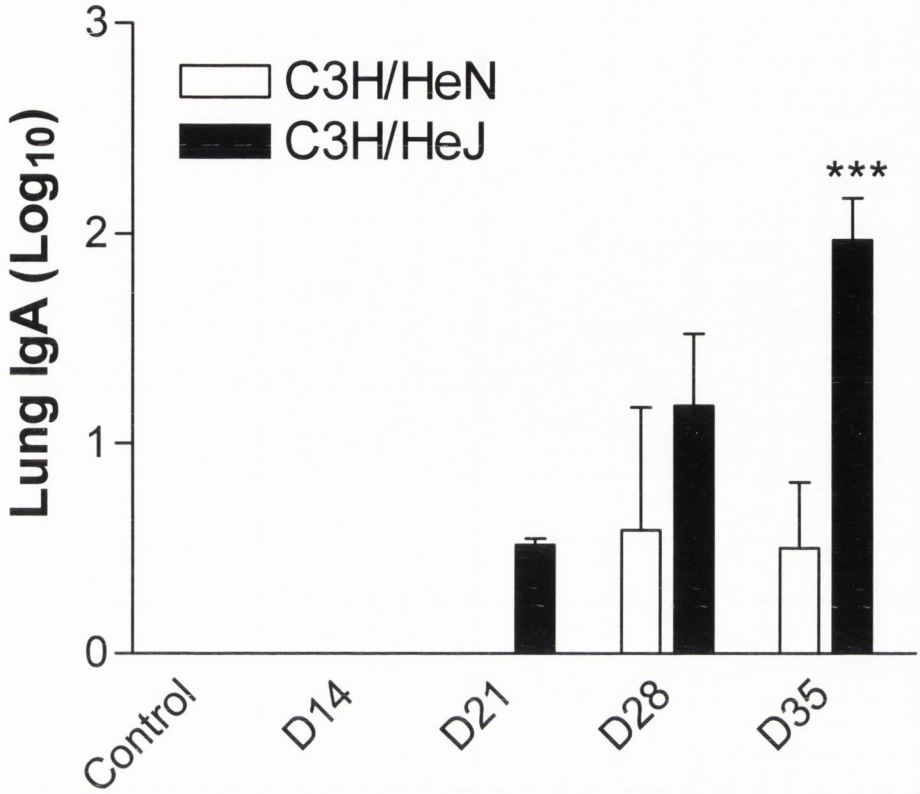


FIGURE 3.14 Enhanced IgA response in the lungs of *B. pertussis* infected C3H/HeJ mice. C3H/HeN and C3H.HeJ mice were aerosol challenged with *B. pertussis*, and groups of four mice were sacrificed 3h, or 3, 7, 14 or 21 days later. Lungs were removed and IgA responses to sonicated *B. pertussis* (5 μg/ml) were evaluated by immunoassay. Results are expressed as mean antibody titres (± SD) for four mice per group. ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

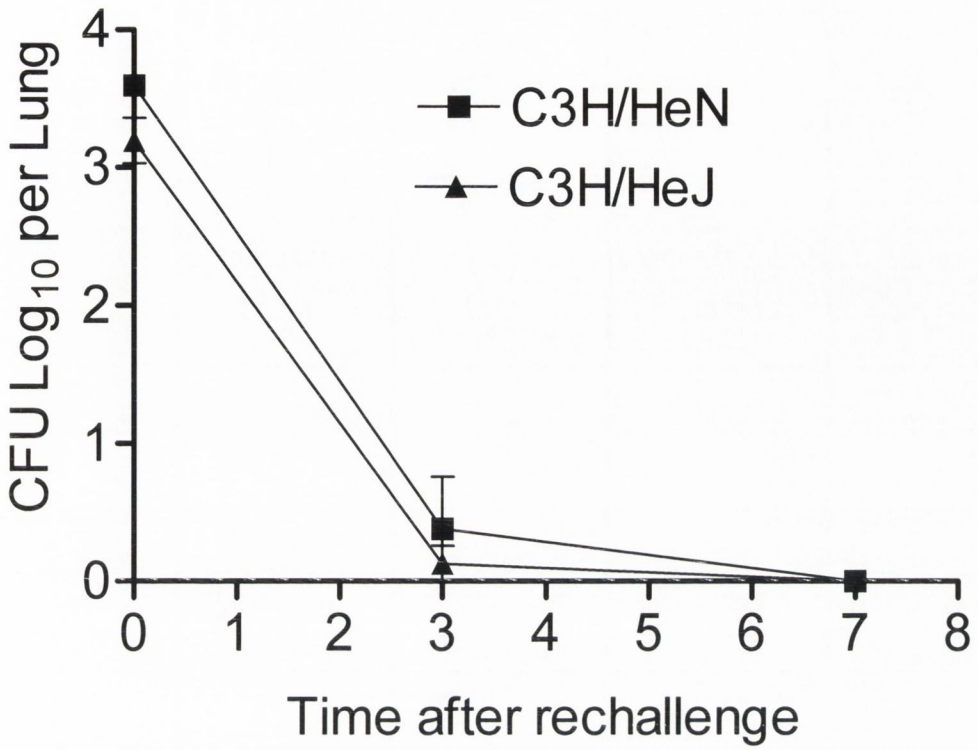


FIGURE 3.15 Rapid clearance of *B. pertussis* in lungs of C3H/HeN and C3H/HeJ mice following *B. pertussis* respiratory rechallenge. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. Groups of four mice were sacrificed 3h, 3, 7, 10, and 14 days after rechallenge. Lungs were aseptically removed and the number of viable *B. pertussis* was determined by performing CFU counts on lung homogenates. Results are mean (± SD) for four mice per group at each time point.

FIGURE 3.16. Early inflammatory cytokine and chemokine responses detected in the lungs of C3H/HeN mice are absent in TLR4-defective mice after *B. pertussis* rechallenge. C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. Cytokine and chemokine analysis was performed on lung homogenates from C3H/HeN and C3H/HeJ mice 3h, or 3, 7, 10, or 14 days after rechallenge. Results are mean (± SD) for four mice per group at each time point. ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

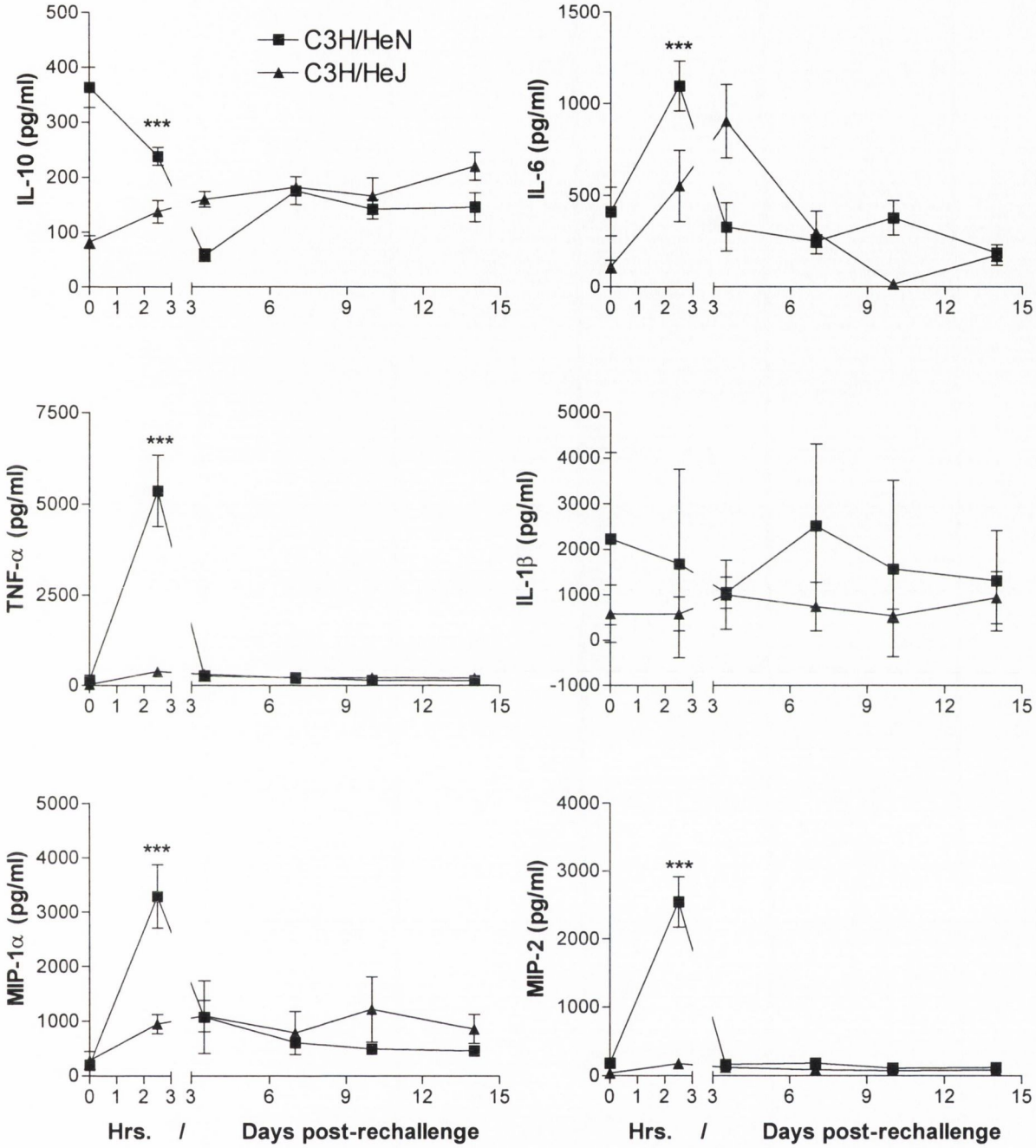

C3H/HeN
C3H/HeJ
IL-10 (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
IL-1β (pg/ml)
MIP-1α (pg/ml)
MIP-2 (pg/ml)
***
Hrs. / Days post-rechallenge

FIGURE 3.17. Enhanced Ag-specific IFN-γ production in TLR4-defective mice following B. pertussis respiratory re-challenge. C3H/HeN and C3H.HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. Spleens from *B. pertussis* re-infected C3H/HeN and C3H/HeJ mice were removed 7 days (A), 10 days (B) and 14 days (C) after rechallenge. Spleen cells were stimulated with *B. pertussis* antigens (FHA 0.25, 1.0 ug/ml; heat inactivated PT 0.25 ug/ml; heat- killed *B. pertussis* 1 x 10^5 and PMA/CD3 or medium as positive and negative controls respectively). Supernatants were removed after 3 days and tested for IFN-γ and IL-10 by immunoassay. Results are mean (± SD) values for five mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

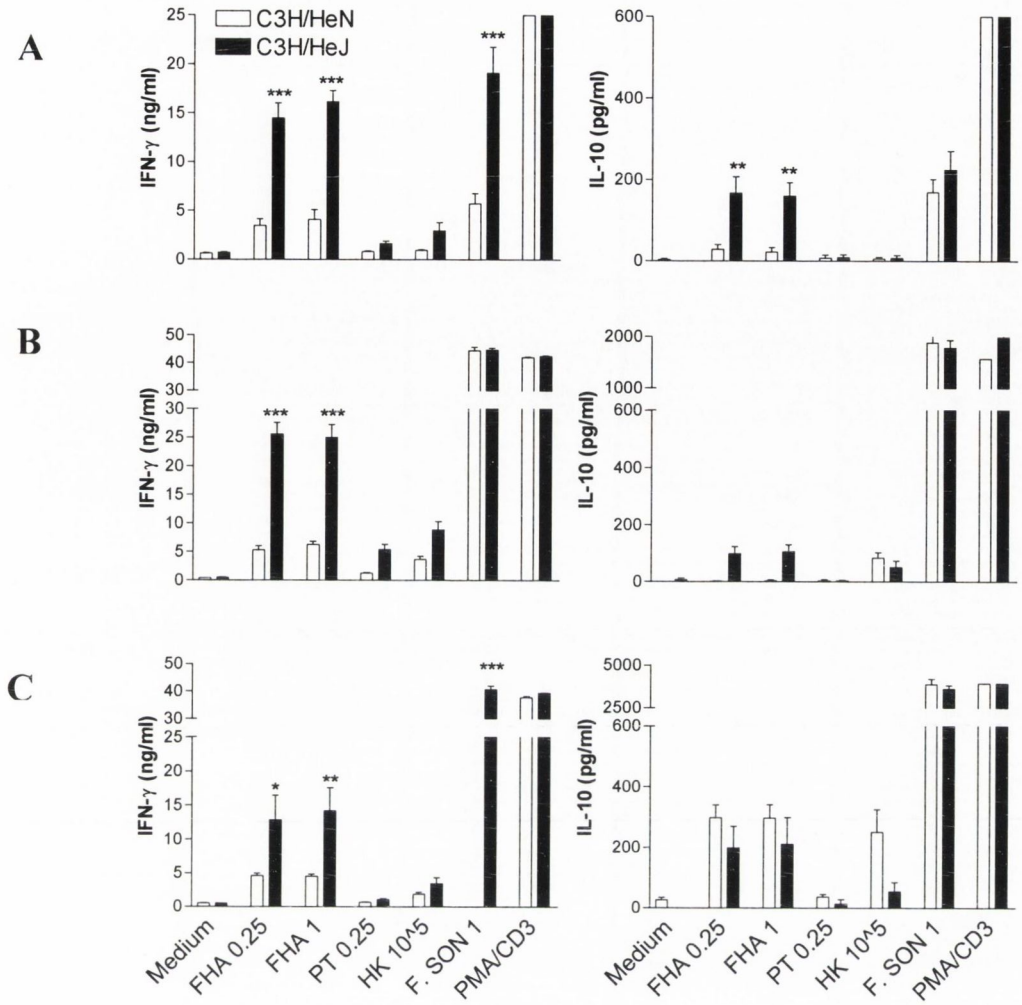
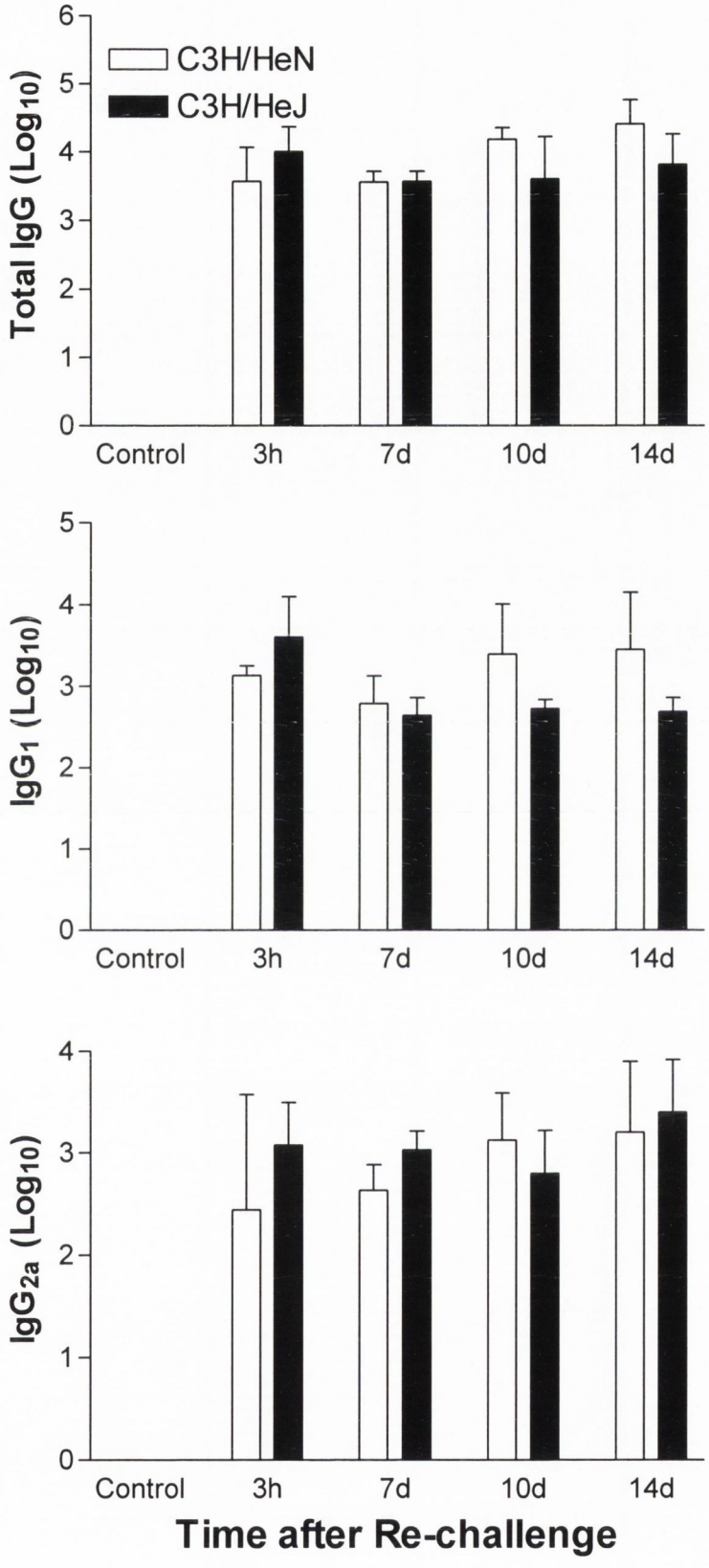

A
B
C
C3H/HeN
C3H/HeJ
IFN-γ (ng/ml)
IL-10 (pg/ml)
Medium
FHA 0.25
FHA 1
PT 0.25
HK 10^5
F. SON 1
PMA/CD3

FIGURE 3.18. Serum antibody responses in C3H/HeN and C3H/HeJ mice after *B. pertussis* re-challenge.

C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. Antibody titres to a *B. pertussis* (sonicated *B. pertussis*, 5 ug/ml) in the sera of both strains of mice were analysed by ELISA 3h, 3, 7,10 and 14 days post re-challenge. Results are expressed as mean antibody titres (± SD) for four mice per group.


C3H/HeN
C3H/HeJ
Total IgG (Log10)
IgG1 (Log10)
IgG2a (Log10)
Control
3h
7d
10d
14d
Time after Re-challenge

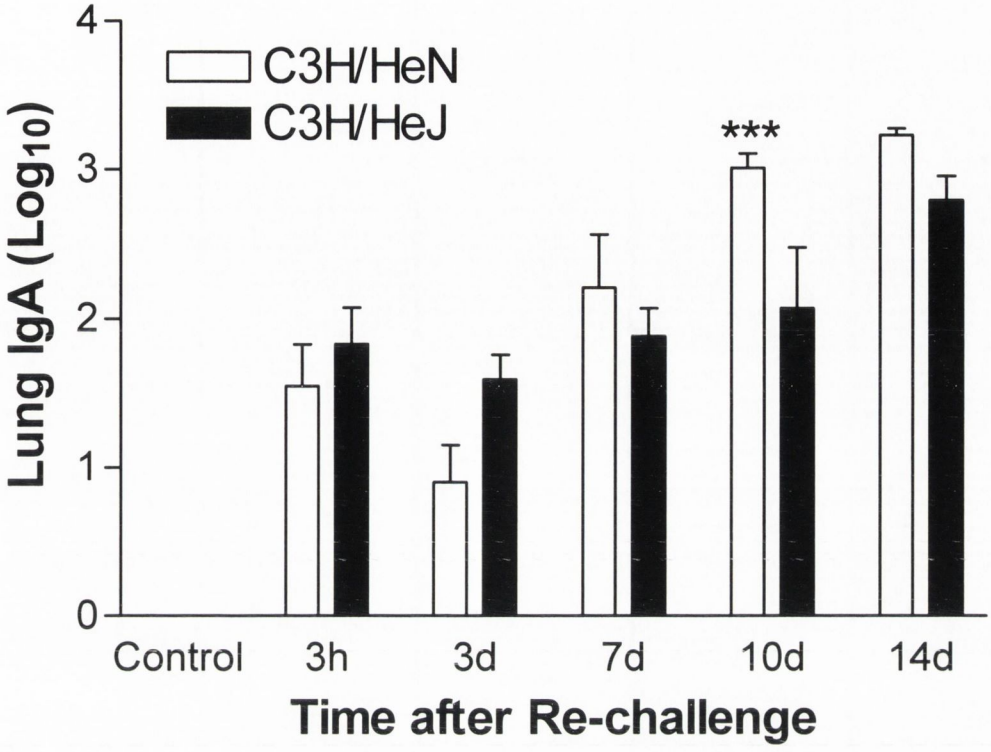


FIGURE 3.19. Similar lung IgA responses in C3H/HeN and C3H/HeJ mice after *B. pertussis* re-challenge. C3H/HeN and C3H.HeJ mice were aerosol challenged with *B. pertussis*, allowed to recover for 4 weeks before exposure to a secondary *B. pertussis* challenge. IgA responses to sonicated *B. pertussis* (5 μg/ml) in the lung were evaluated before challenge and at intervals after challenge. Results are expressed as mean antibody titres (± SD) for four mice per group. ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

Chapter 4

The role of TLR4 in whole cell and acellular pertussis vaccine efficacy

4.1 INTRODUCTION

The development of methods to isolate and culture *Bordetella pertussis* on artificial medium in 1906 led to the development of a vaccine for the prevention of whooping cough, a disease that at the turn of the twentieth century killed more than 5 of every 1000 children born in the United States (Cherry, 1996). Whole cell pertussis vaccines (Pw) were first licensed in the United States in 1914 and became available in a combination vaccine with diphtheria and tetanus toxoids (DTP) in 1948 (Yeh, 2003). Immunisation with Pw although effective at preventing whooping cough, is associated with a number of side effects. These include minor reactions (which occur in about 50% of recipients), such as redness, swelling and pain at the site of injection. Minor systemic reactions, such as fever, irritability and drowsiness, are also common. However, disabling and sometimes fatal reactions have also been reported following Pw administration, such as encephalopathy, or sudden infant death (Cherry, 1996; Cherry and Olin, 1999; Mills, 2001; Yeh, 2003). Concerns regarding the safety of Pw motivated the development of new generation acellular pertussis vaccines (Pa), which are prepared with highly purified antigens from *B. pertussis* (Greco *et al.*, 1996; Olin *et al.*, 1997). Although the safety profile of Pa is considerably higher when compared with Pw, the level of protection induced is generally lower (Mills, 2001).

The mechanisms of protective immunity against *B. pertussis* infection following vaccination remain to be fully elucidated. Research into pertussis vaccine-induced protection has traditionally concentrated on the role of humoral immunity and studies have shown that vaccination with Pw or Pa induces *B. pertussis*-specific antibodies, believed to be required for protection (Sato *et al.*, 1984; Simondon, 1997), although the validity of such serological correlates has been a subject of much debate

(Cherry, 1992; Leef *et al.*, 2000; Storsaeter *et al.*, 1998). There is, however, increasing evidence supporting a central role of cell mediated immunity (CMI) in protection with Pw. Persistence of immunity appears to be associated with CMI rather than humoral responses and it is likely that T cells play a role in immunological memory and long-term protection (Mahon *et al.*, 2000; Mills *et al.*, 1993). An examination of T cell responses in children immunised with Pw or Pa suggested that these vaccines induced distinct subpopulations of $CD4^+$ cells. Several studies demonstrated that Pw selectively induced Th1 cells and that Pa generated T cells with a Th0 or mixed Th1/Th2 cytokine profile (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998). However, there is also evidence to suggest that the Th1-Th2 dichotomy with Pw and Pa in mice may not be so clear cut. *B. pertussis*-specific T cell responses in mice immunised with Pa shifted to a mixed Th1/Th2 profile after respiratory infection with *B. pertussis* (Mahon *et al.*, 2000), and addition of IL-12 to a Pa formulation resulted in the induction of IFN-γ-secreting T cells (Mahon *et al.*, 1996). The addition of IL-12 to Pa was found to enhance Th1 cytokine production and augment its protective efficacy to the level of a potent Pw (Mahon *et al.*, 1996), providing further evidence of a protective role for Th1 cells (Mills, 2001). The presence of endotoxin and active PT in Pw may influence the T cell type induced (Mills, 2001). Data in chapter 3 illustrated that *B. pertussis* LPS is a potent inducer of IL-12p70, a cytokine known to promote the differentiation of Th1 cells.

The discovery that TLR signalling directly activates dendritic cells has had important implications for vaccine design. An essential step in the generation of adaptive immune responses, which mediate vaccine induced protection, is the activation of innate immune responses. Pathogen recognition receptors, especially, TLRs play a key role in innate cell activation. However, the role of innate immunity

and TLR signalling in pertussis vaccine efficacy has not been addressed. This chapter set out to examine the role of TLR4 in mediating the protective properties of Pw and Pa. The findings here demonstrate that TLR4 plays a critical role in the protective immunity elicited by Pw and also mediates some of the protective attributes of Pa.

4.2 RESULTS

4.2.1 Defective Pa and Pw mediated protection against *B. pertussis* in TLR4-defective mice.

A role for TLR4 in mediating the clearance of a primary infection with *B. pertussis* was established in the previous chapter. This study examined the role of TLR4 in protective immunity generated with Pw and Pa. The bacterial load in the lungs after *B. pertussis* challenge of control C3H/HeN and C3H/HeJ mice immunised with PBS was similar to that observed during the primary infection described in chapter 3. TLR4-defective mice had significantly higher bacterial numbers in their lungs ($p < 0.001$) when compared with C3H/HeN mice. Immunisation with Pa elicits protection in both C3H/HeN and C3H/HeJ mice, with a dramatic decline in CFU counts in the lungs after challenge when compared with PBS immunised mice (Fig. 4.1). However, the bacterial burden in lungs of Pa immunised TLR4-defective C3H/HeJ mice was significantly higher ($p < 0.05$, $p < 0.001$) than in the C3H/HeN mice on 7, 10 and 14 days after challenge with *B. pertussis,* demonstrating that TLR4 does play a role in the protective capacity of Pa. The most effective clearance of *B. pertussis* infection is observed in C3H/HeN mice immunised with Pw (Fig. 4.1). A dramatic decline in bacterial numbers is observed in C3H/HeN mice 7 days after challenge and the *B. pertussis* infection is completely cleared by day 10. In contrast, the Pw immunised TLR4-defective C3H/HeJ mice have significantly higher ($p < 0.001$) numbers of *B. pertussis* in their lungs 7, 10 and 14 days after infection. Pw elicits very little protection in the TLR4-defective mice with an approximate one $\log_{10}$ reduction in the CFU counts observed in the lungs of Pw- immunised C3H/HeJ mice

when compared with PBS-immunised C3H/HeJ mice 3-14 days after *B. pertussis* challenge. This suggests that TLR4 is central to the protective immunity elicited by Pw.

4.2.2 Differential cytokine and chemokine production in the lungs of C3H/HeN and C3H/HeJ mice immunised with PBS, Pa or Pw prior to and following infection with *B. pertussis*.

To further investigate the local immune response to *B. pertussis* in the lungs of immunised mice, lungs from PBS-, Pa- and Pw- immunised C3H/HeN and C3H/HeJ mice were examined for cytokine and chemokine production before and after infection with *B. pertussis*. The protective capacities of both Pa and Pw reflected a lower inflammatory response in the lungs after *B. pertussis* challenge of Pa- and Pw-immunised C3H/HeN and C3H/HeJ mice when compared with PBS-immunised control mice (Fig. 4.2A and 4.2B). This is particularly evident with IL-6 (Fig. 4.2A) and TNF-α (Fig. 4.2B) production. Consistent with previous findings, higher concentrations of IL-6 (Fig. 4.2A), TNF-α, MIP-1α and MIP-2 (Fig. 4.2B) are observed in the lungs of PBS-immunised C3H/HeN mice compared with TLR4-defective C3H/HeJ mice after infection with *B. pertussis*. However, significantly higher IL-6 ($p < 0.05$), TNF-α ($p < 0.01$), MIP-1α ($p < 0.001$) and MIP-2 ($p < 0.001$) are observed in the lungs of TLR4-defective mice at 10 and/or 14 days after *B. pertussis* challenge. The production of the Th1 promoting cytokine, IL-12p70, is transient but significantly higher ($p < 0.05$) in the lungs 3 days after infection with *B. pertussis* in C3H/HeN mice compared with C3H/HeJ mice immunised with either Pa or Pw. The concentrations of IFN-γ in the lungs of Pa and Pw immunised mice were

low and there was no difference between immunised C3H/HeN and C3H/HeJ mice at the timepoints examined. A dramatic difference in local IL-10 production was observed in the lungs of C3H/HeN and C3H/HeJ mice immunised with Pa. Significantly elevated IL-10 production ($p < 0.001$) was observed in the lungs of Pa-immunised mice 7 and 10 days after *B. pertussis* challenge of C3H/HeN mice, when compared with TLR4-defective mice, providing further evidence to that shown in chapter three, that innate and adaptive IL-10 production in response to *B. pertussis* is dependent on TLR4.

4.2.3 Reduced antigen-specific IL-10 production in TLR4-defective mice immunised with Pa and Pw.

To further elucidate the role that TLR4 may have in the protective capacities of Pa and Pw, *B. pertussis*-specific T cell responses were examined in Pa and Pw immunised mice prior to *B. pertussis* challenge. C3H/HeN and C3H/HeJ mice were immunised intraperitoneally at 0 and 4 weeks with PBS, Pa or Pw. Two weeks after the second immunisation, spleen cells were isolated from immunised mice and restimulated *ex vivo* with *B. pertussis* antigens. A significantly lower proliferation response to *B. pertussis* (formalin-sonicate) was observed in spleen cells from Pa-immunised TLR-4 defective mice when compared with C3H/HeN mice ($p < 0.001$) (Fig. 4.3). Proliferation of *B. pertussis*-specific T cells from Pw immunised C3H/HeJ mice was also significantly reduced ($p < 0.05$) when compared with C3H/HeN mice. These results indicate that TLR4 plays a role in the quality of the adaptive response elicited by both Pa and Pw.

Immunisation of mice with Pw has been shown to selectively prime Th1 cells characterised by the production of IFN-γ and low concentrations of IL-4 and IL-5 (Redhead *et al.*, 1993), whereas immunisation with Pa has been associated with the production of IL-4, IL-5 and low concentrations of IFN-γ (Mills *et al.*, 1998). Consistent with these findings, immunisation with Pw induced *B. pertussis*-specific T cells that secreted high levels of IFN-γ in both the C3H/HeN and the C3H/HeJ mice (Fig. 4.4). Although *B. pertussis* IFN-γ appears slightly enhanced in the TLR4-defective mice, there was no significant difference in antigen-specific IFN-γ production between the Pw-immunised C3H/HeN and C3H/HeJ mice (Fig. 4.4). The data in chapter three showed that *B. pertussis*-specific IL-10 production was significantly reduced in the C3H/HeJ mice compared with C3H/HeN mice at 14 and 21 days after infection with *B. pertussis*. Similarly, in this study, *B. pertussis*-specific IL-10 production was found to be significantly lower in spleen cells from Pa- and Pw-immunised C3H/HeJ mice compared with C3H/HeN mice 14 days after challenge (Fig. 4.4). FHA-specific spleen cell IL-10 production is also lower (although not significantly) in Pa immunised C3H/HeJ compared with C3H/HeN mice. Immunisation of C3H/HeN and C3H/HeJ mice with Pa induced antigen-specific IL-5 production. Although, FHA-specific IL-5 production was lower in spleen cells from the Pa immunised C3H/HeJ mice, the response was not significantly different from that seen with spleen cells from the C3H/HeN mice. Low concentrations of antigen-specific IL-4 production were observed in both Pa and Pw immunised C3H/HeN and C3H/HeJ mice.

4.2.4 Similar *B. pertussis*-specific antibody responses in Pa or Pw immunised C3H/HeN and C3H/HeJ mice prior to and 14 days after *B. pertussis* infection.

Both Pa and Pw induce high levels of specific Abs directed against several *B. pertussis* virulence factors. Although the mechanisms by which Abs contribute to host protection is still unclear, several studies have provided evidence that Ab concentrations in serum correlate with protection against infection in children (Cherry *et al.*, 1998; Hewlett and Halperin, 1998; Mahon *et al.*, 1997; Rodriguez *et al.*, 2001; Rodriguez and van der Pol, 2003; Storsaeter *et al.*, 1998). Studies in mice have shown that in general, Pw induces more IgG2a than IgG1, whereas Pa induces more IgG1 than IgG2a (Mills, 2001). Infection with *B. pertussis* has also been shown not to greatly enhance Ab titres in immunised mice (Mills, 2001). Immunisation with Pa or Pw induced similar IgG titres against *B. pertussis*, FHA and PT when tested before and 14 days after *B. pertussis* respiratory challenge (Fig. 4.5A). *B. pertussis*-specific sera IgG1 was significantly higher ($p < 0.001$) 14 days after *B. pertussis* infection in C3H/HeJ mice compared with C3H/HeN mice immunised with Pa (Fig. 4.5B (i)). In contrast, the PT-specific IgG1 response was significantly reduced ($p < 0.001$) in the Pa immunised C3H/HeJ mice when compared with C3H/HeN mice prior to *B. pertussis* challenge (Fig. 4.5B (iii)). The IgG1 response against all the *B. pertussis* antigens examined was similar in both the C3H/HeN and C3H/HeJ mice immunised with Pw (Fig. 4.5B).

Immunisation with Pw is associated with the induction of strong IgG2a responses (Mills *et al.*, 1998). Both C3H/HeN and C3H/HeJ mice immunised with Pw induced a similar IgG2a response to *B. pertussis*, FHA and PT (Fig. 4.5B).

However, the FHA-specific IgG2a response was significantly lower ($p < 0.05$) in the TLR4-defective mice 14 days after *B. pertussis* infection. No *B. pertussis*-specific IgG2a was detected in the sera of Pa immunised C3H/HeN mice (Fig. 4.5B (i)). In comparison, significant quantities ($p < 0.001$) of *B. pertussis* IgG2a was induced by Pa immunised C3H/HeJ mice. This is consistent with reduced IL-10 and enhanced Th1 response observed in *B. pertussis* infected TLR4-defective mice.

4.2.5 Similar IgA titres in the lungs of Pa and Pw immunised C3H/HeN mice and C3H/HeJ mice after infection with *B. pertussis*.

IgA represents the most prevalent Ig isotype at mucosal sites. It has been reported that IgA can induce effective phagocytosis of *B. pertussis* via FcαR1 (Hellwig *et al.*, 2001). Given the relevance of IgA in mediating protection to *B. pertussis*, the kinetics of IgA production was examined in the lungs of Pa and Pw immunised C3H/HeN and C3H/HeJ mice before and after respiratory challenge with *B. pertussis*. Similar IgA titres were observed in PBS, Pa and Pw immunised C3H/HeN and C3H/HeJ mice 3, 7 and 14 days after infection with *B. pertussis*. These results suggest that a TLR4 defect does not negatively impact on *B. pertussis*-specific IgA induced by Pa or Pw.

4.2.6 Components of both Pa and Pw induce pro- and anti- inflammatory cytokine production by signalling through TLR4.

Both Pa and Pw failed to elicit the same degree of protection in the TLR4-defective mice when compared with the C3H/HeN mice (Fig. 4.1). An important step in the induction of an adaptive immune response to a vaccine is the activation of APCs, such as DCs. Therefore, Pa and Pw were tested for their ability to activate DC *in vitro*. Bone-marrow-derived DC from C3H/HeN and C3H/HeJ mice were stimulated with a range of dilutions of Pa and Pw and cytokine concentrations were measured 18 h later. The Pa used in this study was a two component PT-FHA pertussis vaccine and was assigned a potency of 40 IU by the manufacturer (using a Japanese modified Kendrick test). The Pw used in this study was a thiomersal killed *B. pertussis* vaccine and was assigned a potency of 46 IU per ampoule (Table 4.1). Protein concentrations in the preparations could not be measured accurately by a BSA assay due to other components in the vaccine such as preservatives. Therefore, a range of dilutions of each vaccine was used to stimulate the DCs. The starting dilution of 1/5 was equivalent to 0.61 IU/ml for the Pw and 0.53 IU/ml for the Pa.

Pw stimulated a range of pro- and anti-inflammatory cytokine production from DC. Very high concentrations of IL-1β, TNF-α, IL-12p40 and IL-10 were detected in DC derived from C3H/HeN mice stimulated with Pw. Pw at a dilution as high as 1/320 (equivalent to 0.00..) induced high levels of IL-1α, TNF-α, Il-6 and Il-12p40. In contrast, IL-10 was detected only at lower dilutions. Pw-induced production of IL-1β, TNF-α, IL-12p40 and the anti-inflammatory cytokine, IL-10 were significantly reduced ($p < 0.01$-$p < 0.001$) in DC derived from TLR4-defective mice. A significant reduction ($p < 0.05$) in IL-6 production from C3H/HeJ DC was not observed until a

1/160 dilution of Pw, suggesting that components of Pw induce IL-6 production independently of TLR4 mediated signalling (Fig. 4.7). Pa was less potent than Pw in the induction of cytokine production from DCs. The production of IL-1β, TNF-α, and IL-12p40 was detected from C3H/HeN DCs with 1/5 and 1/10 dilutions of Pa (Fig. 4.7). Similar to Pw, a significant reduction ($p < 0.001$) in cytokine production was observed in DCs derived from TLR4-defective mice stimulated with Pa. Pa failed to induce IL-10 production from DCs at all dilutions examined (Fig. 4.7).

The reduction in cytokine production induced by Pa and Pw from DC from TLR4-defective mice, indicates that both vaccines contained LPS or other TLR4 ligands. Therefore, the LPS content of Pa and Pw was quantified by an LAL assay. The LPS content of Pw was found to be 133450 EU/ml (equivalent to 177.93 μg/human dose) (Table 4.1). Pa contained 6.96 EU/ml (equivalent to 1.44 ng/human dose). These results indicate that activation of DC cytokine production by both Pa and Pw is largely mediated by TLR4 signalling, probably due to contaminating LPS in the vaccines.

TABLE 4.1 Description and Endotoxin content of Pa and Pw.

VACCINE	REF. NO.	SOURCE	DESCRIPTION	COMPOSITION	CONC. OF ENDOTOXIN (EU/ML)*	EU/MOUSE DOSE (0.8 IU/300μL)	EU/HUMAN DOSE (4 IU/500μL)
Whole Cell Pertussis (Pw)	66/303	NIBSC	Freeze-dried whole cell pertussis vaccine	2 ml aliquots of an aqueous soln which contained 10 litres of bacterial suspension -assigned a potency of 46 IUs per ampoule	133450 EU/ml (equivalent to 13345 ng/ml)	35586.7 EU (equivalent to 3558.7 ng)	177933.5 EU (equivalent to 17793.4 ng)
Acellular Pertussis (Pa)	JNIH-3	NIBSC	2-component freeze-dried acellular pertussis vaccine	7.5 μg PT 7.5 μg FHA -assigned a potency of 40 IUs per ampoule (by Japanese modified Kendrick test)	6.96 EU/ml (equivalent to 696 pg/ml)	2.09 EU (equivalent to 209 pg)	10.44 EU (equivalent to 1044 pg)

* **1 EU = 100 pg/ml**

4.3 DISCUSSION

Vaccines, including the pertussis Pa and Pw protect against infection by inducing adaptive immune responses, which are also dependent on innate cell activation. The discovery of TLRs has brought a greater appreciation of how the innate immune system initiates, activates and directs antigen-specific T cell and antibody responses. Most licensed vaccines, particularly live, attenuated or killed whole cell vaccines (e.g. Pw) contain components (i.e. PAMPs) that can activate innate immune responses. However, current trends in vaccine development are to move away from killed attenuated vaccines towards safer and better defined subunit vaccines, produced as highly purified recombinant components (O'Hagan and Valiante, 2003). These purified recombinant antigens are generally well tolerated, but often lack components including PAMPs that are required for optimal innate immune activation, thereby rendering subunit vaccines less efficient at inducing a protective adaptive immune response (Valiante *et al.*, 2003). The introduction of Pw in 1914 brought great success in reducing the incidence of pertussis by unwittingly exploiting the innate immune system. However, concerns regarding the safety of Pw led to the development of a less reactogenic, but safer, Pa (Mills, 2001). The role of innate immunity in the protection elicited by Pw and Pa has until this study received little attention.

This study compared the protective capacities of Pa and Pw and examined the role of TLR4 in mediating the efficacy of each vaccine. The significant finding is that TLR4 is essential for inducing the full protective effect of Pw but is less important in mediating the protective capacity of Pa, and that these effects may reflect the relative levels of LPS in the respective vaccines.

The mechanisms underlying the ability of both Pa and Pw to elicit protection remain to be fully elucidated. Murine models, however, have played a major role in advancing our current knowledge of immunity to pertussis. It has previously been demonstrated that the rate of *B. pertussis* clearance following respiratory challenge of immunised mice correlated with vaccine efficacy in children (Mills *et al.*, 1998). There have been few attempts to examine the role of the innate immune system in pertussis vaccine induced immunity and the role of TLRs has not been addressed. The present study demonstrates an absolute requirement for TLR4 in mediating protection against *B. pertussis* by immunisation with Pw in mice. Although protection was observed in TLR4-defective mice immunised with Pa, this protection was sub-optimal when compared to that seen in C3H/HeN mice immunised with Pa. The results suggest that an attenuated innate response to both Pw and Pa in the TLR4-defective mice compromises the protective capacity of these vaccines.

The production of inflammatory mediators and recruitment of immune effector cells to the site of infection is an important feature of protective cellular immune responses. A study by McGuirk and Mills (2000) concluded that the recruitment and activation of inflammatory cells, such as neutrophils, into the lungs following *B. pertussis* challenge may play a pivotal role in facilitating the early resolution of infection following immunisation with Pw. This study and others also established that protection induced by Pa was mainly antibody mediated and associated with reduced inflammatory responses (McGuirk and Mills, 2000; Mills *et al.*, 1998). In chapter three, the defective innate response to *B. pertussis* in cells from TLR4-defective mice *in vitro* was reflected in an impaired inflammatory cytokine response in the lungs of C3H/HeJ mice soon after *B. pertussis* primary infection. The transient peak of IL-1β, TNF-α, MIP-1α and MIP-2 observed in the lungs of

C3H/HeN mice within 3 h of *B. pertussis* challenge was not detected in the C3H/HeJ mice, demonstrating that the initial inflammatory response is much reduced in these TLR4-defective mice. These inflammatory mediators play important roles in leukocyte recruitment in the protective cellular response to bacterial infections (McGuirk and Mills, 2000; Mizgerd *et al.*, 2001; Standiford *et al.*, 1996). Three days after *B. pertussis* infection the inflammatory response in the lungs of Pa- and Pw-immunised mice has decreased considerably compared with the PBS-immunised control mice, suggesting that any inflammatory response induced in the lungs by Pa or Pw is absent or possibly occurred earlier than the first timepoint examined. Overall, the protective capacities of both vaccines were evident by the reduced inflammatory response in the lungs of Pa and Pw immunised mice when compared with the PBS-immunised control mice at 3, 7,10 and 14 days after infection with *B. pertussis*. High concentrations of local IL-10 production were detected in the lungs of Pa-immunised C3H/HeN mice 7 and 10 days after infection with *B. pertussis*. However, IL-10 is absent from the lungs of the TLR4-defective mice at these timepoints. FHA, a component of the Pa used has been shown to induce IL-10 production from both DC and macrophages (McGuirk *et al.*, 2002; McGuirk and Mills, 2000). Although, this study has not established the source of IL-10 in the lungs of Pa immunised C3H/HeN mice, it would appear that the IL-10 is induced by a TLR4-dependent mechanism and most likely involves FHA and LPS.

An examination of the T cell responses in children immunised with Pw and Pa suggested that these vaccines induced distinct populations of $CD4^+$ T cells. Several studies have concluded that Pw mainly induce Th1 cells and that Pa generate T cells with a mixed Th1/Th2 cytokine profile (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998). Similarly, in the murine model, immunisation with Pw selectively

primes Th1 cells, whereas immunisation with Pa generated T cells that secreted IL-4, IL-5 and low IFN-γ (Redhead *et al.*, 1993). This bias in $CD4^+$ T cell differentiation appears to affect the quality of the protective immune response elicited by Pw- or Pa-immunised mice and humans (Mills, 2001). A high concentration of *B. pertussis*-specific IFN-γ production, indicative of a Th1 response, was detected in antigen stimulated spleen cells from C3H/HeN and C3H/HeJ mice immunised with Pw. Consistent with previous studies which reported antigen-specific IL-10 production in Pw immunised mice (Ennis *et al.*, 2005; van den Berg *et al.*, 2000), Antigen-specific IL-10 was detected in spleen cells from C3H/HeN mice immunised with Pw. Importantly, a significant reduction in *B. pertussis*-specific IL-10 was evident in TLR4-defective mice immunised with Pw. This is consistent with the antigen-specific T cell response observed in the TLR4-defective mice following infection with *B. pertussis* (chapter 3). FHA-specific and *B. pertussis*-specific IL-10 production was also reduced in TLR4-defective mice when compared to C3H/HeN mice immunised with Pa. This suggests that the absence of functional TLR4 compromises the ability of the innate immune system to produce IL-10 and thereby results in defective IL-10 production, which was shown as important in the control of inflammation caused by *B. pertussis* infection (chapter 3).

Antibodies have a central role in protection against *B. pertussis* (Bruss *et al.*, 1999; Granstrom *et al.*, 1991; Mills, 2001), where they may function either by neutralising bacterial toxins, inhibiting extracellular bacteria from binding to cells in mucosal tracts or by promoting bacterial uptake and destruction by macrophages and neutrophils (Mills, 2001). Clinical trials with Pa, including ones with lower efficacy vaccines, reported high levels of antibodies against the antigen components of each of the vaccines early after immunisation (Greco *et al.*, 1996; Gustafsson *et al.*, 1996;

Olin *et al.*, 1997; Simondon *et al.*, 1997; Trollfors *et al.*, 1995). Furthermore, immunisation with Pw also induces antibody responses against individual antigens, although these were more variable, in particular against PT, and this appeared to reflect the variable presence of these components in the vaccine (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Mills, 2001; Olin *et al.*, 1997; Simondon *et al.*, 1997; Yeh, 2003). The present study confirmed that Pa and Pw induced antibodies of distinct specificities and subclass; IgG1 levels were strong in Pa-immunised mice and as expected FHA and PT were major targets for these antibodies. However, the TLR4 defect did not dramatically affect antibody production in mice immunised with Pa or Pw except that levels of IgG2a were higher in TLR4-defective mice immunised with Pa.

As professional APCs, DCs provide a crucial bridge between innate and adaptive immune responses (Banchereau *et al.*, 2000b; Cella *et al.*, 1997). DCs and other APCs (e.g. macrophages) use PRRs such as TLRs, expressed on their cell surface to detect infectious agents. This provides a mechanism by which the immune system initiates both innate and as a consequence, adaptive immunity to infection or following vaccination (O'Hagan and Valiante, 2003). The DCs from TLR4-defective mice had defective IL-1β, TNF-α and IL-12p40 responses to Pw and Pa. FHA has been shown to stimulate IL-10 production from DCs (McGuirk *et al.*, 2002). However, despite the presence of this component, Pa did not induce IL-10 production from either C3H/HeN or C3H/HeJ- derived DCs. The FHA in this vaccine was treated with gluteraldehyde and adsorbed to alum and this may have affected its ability to activate IL-10 production. Pw and to a lesser extent Pa are contaminated with LPS and this is consistent with a previous study (Geier and Geier, 2002). Although, this contaminating LPS is at a relatively low concentration when compared

to that present in the Pw, it may improve the efficacy of the vaccine by acting as an adjuvant or possibly as an antigen. This LPS content may also explain the reduced efficacy of the vaccine in TLR4-defective mice. PT is a major virulence factor of *B. pertussis* and is regarded as a necessary constituent of all Pa preparations (Edwards and Karzon, 1990; Yeh, 2003). PT has a classical AB bacterial exotoxin structure (Tamura *et al.*, 1982). A recent study showed that both the intact and detoxified PT synergised with LPS to promote IL-12 production from human DCs (Ausiello *et al.*, 2002). Synergy between the second Pa component, FHA, and LPS has also been reported. A study using human macrophage-like U937 cells suggested synergy between FHA and LPS for TNF-α production (Abramson *et al.*, 2001). The high LPS content of Pw has been well documented and has been associated with the increased reactogenicity of the vaccine (Donnelly *et al.*, 2001; Geier and Geier, 2004; Sidey *et al.*, 1989). The LPS content of each vaccine appears crucial in activating DCs and consequently, the innate immune response.

Immunological memory is the cornerstone of successful vaccination. The previous chapter established that the memory response to *B. pertussis* was not affected in the C3H/HeJ mice. A recent study identified necrotic cells as potent inducers of NF-κB. Furthermore, the authors demonstrated that necrotic cell-induced NF-κB was dependent on TLR2 signalling pathways (Li *et al.*, 2001). It was hypothesised that innate immunity to *B. pertussis* was triggered in the TLR4-defective mice only after extensive damage in the lungs led to necrotic cell death and consequently TLR2-mediated activation of protective immunity (chapter 3). The reduced protective capacity of Pw in the C3H/HeJ mice suggests that the memory response to *B. pertussis* was compromised. Since memory was determined by the primary immune response to Pw, the ineffectiveness of the vaccine in the TLR4-

defective C3H/HeJ mice may be due to the fact that Pw administration did not elicit damage and necrotic cell death. Therefore, the TLR2 'trigger' was absent and the immune response to Pw and subsequently the memory response to *B. pertussis* was compromised in the C3H/HeJ mice.

Despite clear evidence that pertussis vaccines can protect children from severe whooping cough, the specific immune response that contributes to the protection of these vaccines remains unclear (Mills, 2001; Yeh, 2003). This study has established an absolute requirement for TLR4 in mediating bacterial clearance in mice immunised with Pw and found an attenuated protective capacity of Pa in TLR4-defective mice. In an attempt to elucidate the immunological basis behind these observations, this study examined the cytokine milieu in the lungs, the antigen-specific T cell responses and antibody responses in immunised mice before and after challenge with *B. pertussis*. Apart from the defective antigen-specific IL-10 production by spleen cells from the Pa and Pw immunised C3H/HeJ mice, very little differences were observed in the adaptive responses induced by Pa or Pw TLR4-defective mice compared with C3H/HeN mice before and after infection with *B. pertussis*. However, the *in vitro* response of DC derived from C3H/HeN and TLR4-defective mice stimulated with Pa or Pw suggested an important role for the innate immune response to vaccination. While Pw is comprised of approximately 3000 *B. pertussis* proteins (Geier and Geier, 2004), the response to Pw was severely compromised in DC derived from TLR4-defective mice. The Pa also induced pro-inflammatory cytokine production from DC and this was compromised in DC from TLR4-defective mice, suggesting a role for the contaminating LPS or other TLR4-binding ligands.

One explanation for the significantly reduced protective efficacy of Pw-immunised mice may be a reduced ability of macrophage from TLR4-defective mice

to kill *B. pertussis*. Recently, it has become clear that *B. pertussis* can exploit both extracellular and intracellular niches during infection (Mills, 2001). *B. pertussis* has been shown to persist in murine and rabbit macrophages (Cheers and Gray, 1969; Saukkonen *et al.*, 1991). The ability of *B. pertussis* to invade and survive within human macrophages has also been described (Friedman *et al.*, 1992). These observations suggest that intracellular killing in macrophages may be an important feature of the protective immune response against *B. pertussis* infection (Mills, 2001). IFN-γ has been shown to enhance the ability of macrophages to kill *B. pertussis in vitro* (Mahon and Mills, 1999; Torre *et al.*, 1996). The IFN-γ production by antigen-specific T cells in the spleen appeared similar in Pw-immunised C3H/HeN and C3H/HeJ mice. However, synergy between IFN-γ and TNF-α has been described in the activation of the antibacterial function of macrophages (Kaufmann and Flesch, 1990; Leenen *et al.*, 1994). Although the present study did not examine the macrophage killing in Pw-immunised mice, decreased TNF-α was observed in DC from C3H/HeJ DC stimulated with Pw. An intracellular niche for *B. pertussis* in neutrophils has also been described (Weingart and Weiss, 2000). However, it has been reported that *B. pertussis* is rapidly killed in human neutrophils (Lenz *et al.*, 2000). Therefore, neutrophils are also an important part of the immune defences against *B. pertussis*. Mulligan and Ward (1992) demonstrated that TNF-α and IL-1β are both essential for neutrophil migration to the lungs (Mulligan and Ward, 1992). TNF-α and IL-1β production were shown to be compromised in DC derived from TLR4-defective mice stimulated with Pw. Therefore, a failure to effectively stimulate the antibacterial activity of macrophages and inadequate recruitment of neutrophils may have contributed to the reduced efficacy of the Pw vaccine in TLR4-defective mice.

It is also possible that insufficient damage elicited by *B. pertussis* infection, in the absence of TLR4-signalling may explain the ineffectiveness of Pw in the C3H/HeJ mice. It was hypothesised that innate immunity to *B. pertussis* was only triggered in the TLR4-defective mice after extensive damage in the lungs led to necrotic cell death and consequently TLR2-mediated activation of protective immunity. The limited protection generated by the Pw in the TLR4-defective mice may have resulted in less damage in the lungs, therefore, the TLR2 'trigger' was absent and the *B. pertussis* infection persisted. Further studies are required to investigate this possibility.

Overall, the poor efficacy of Pw in the TLR4-defective mice suggests that LPS contributes to the efficacy of Pw by behaving as an adjuvant as well as an immunomodulator molecule that activates immune cells. Protection with Pa was only marginally reduced in the TLR4-defective mice suggesting that the alum adjuvanted vaccine is not severely affected by the TLR4 defect. The modest reduction in efficacy of Pa in TLR4-defective mice may have resulted from a small contribution of contaminating LPS to the protection efficacy.

In conclusion, this study has established that TLR4 underpins the protective mechanisms of Pw and that TLR4, also mediates some of the protective properties of Pa. The findings of this study also suggest that the LPS component of Pw, which is largely associated with its reactogenicity, makes a vital contribution to the vaccine's efficacy.

Figure 4.1: Reduced efficacy of Pw and Pa in TLR4-defective mice. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at 0 and 4 weeks with $1/5^{th}$ of a human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization, mice were challenged with *B. pertussis* and groups of five mice were sacrificed 3h, or 3, 7, 10, or 14 days later. Lungs were removed and the number of viable *B. pertussis* was determined by performing CFU counts on individual lung homogenates. Results are expressed as mean (± SD) viable *B. pertussis* counts for 5 mice per group. *, $p< 0.05$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

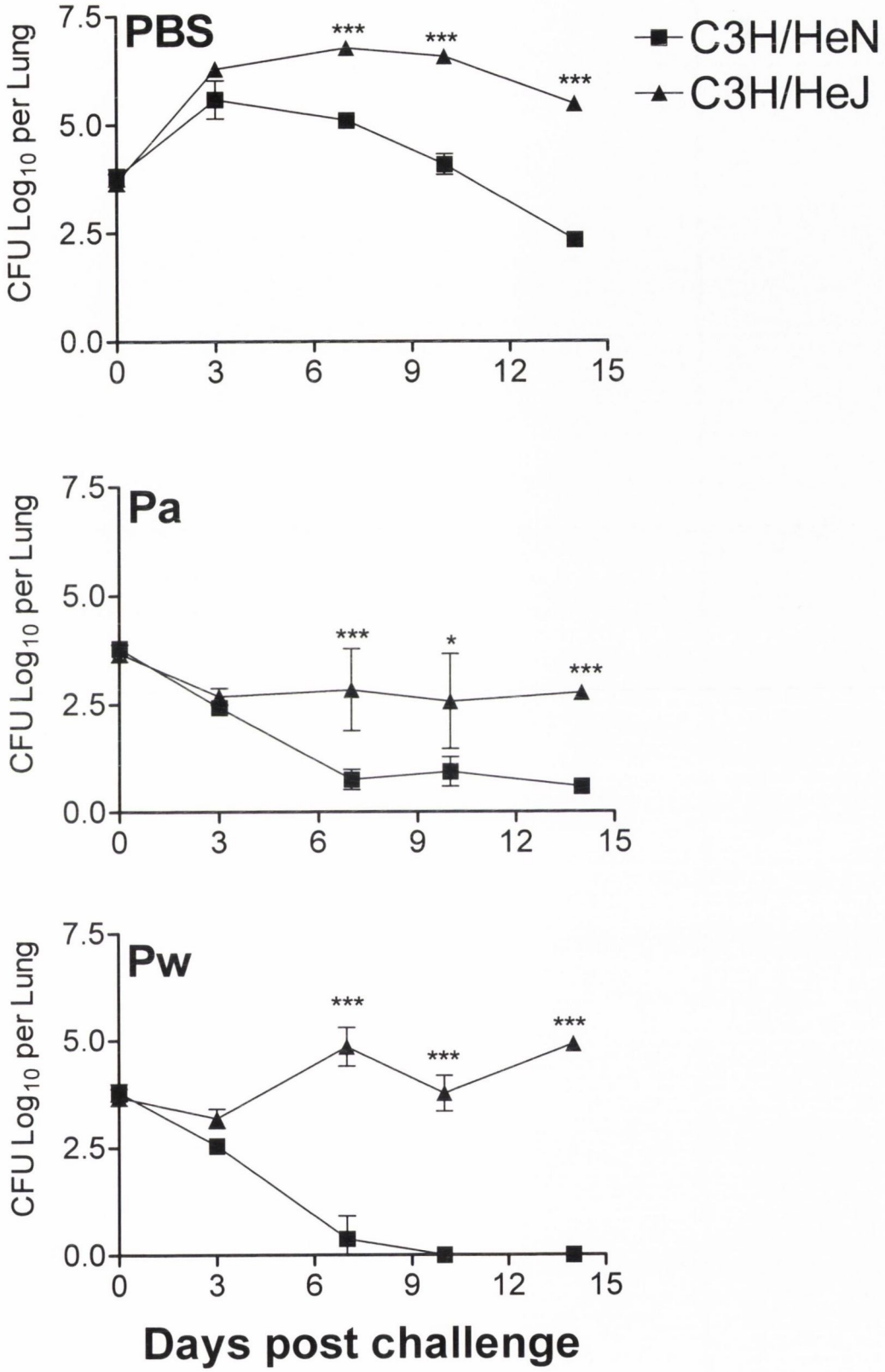
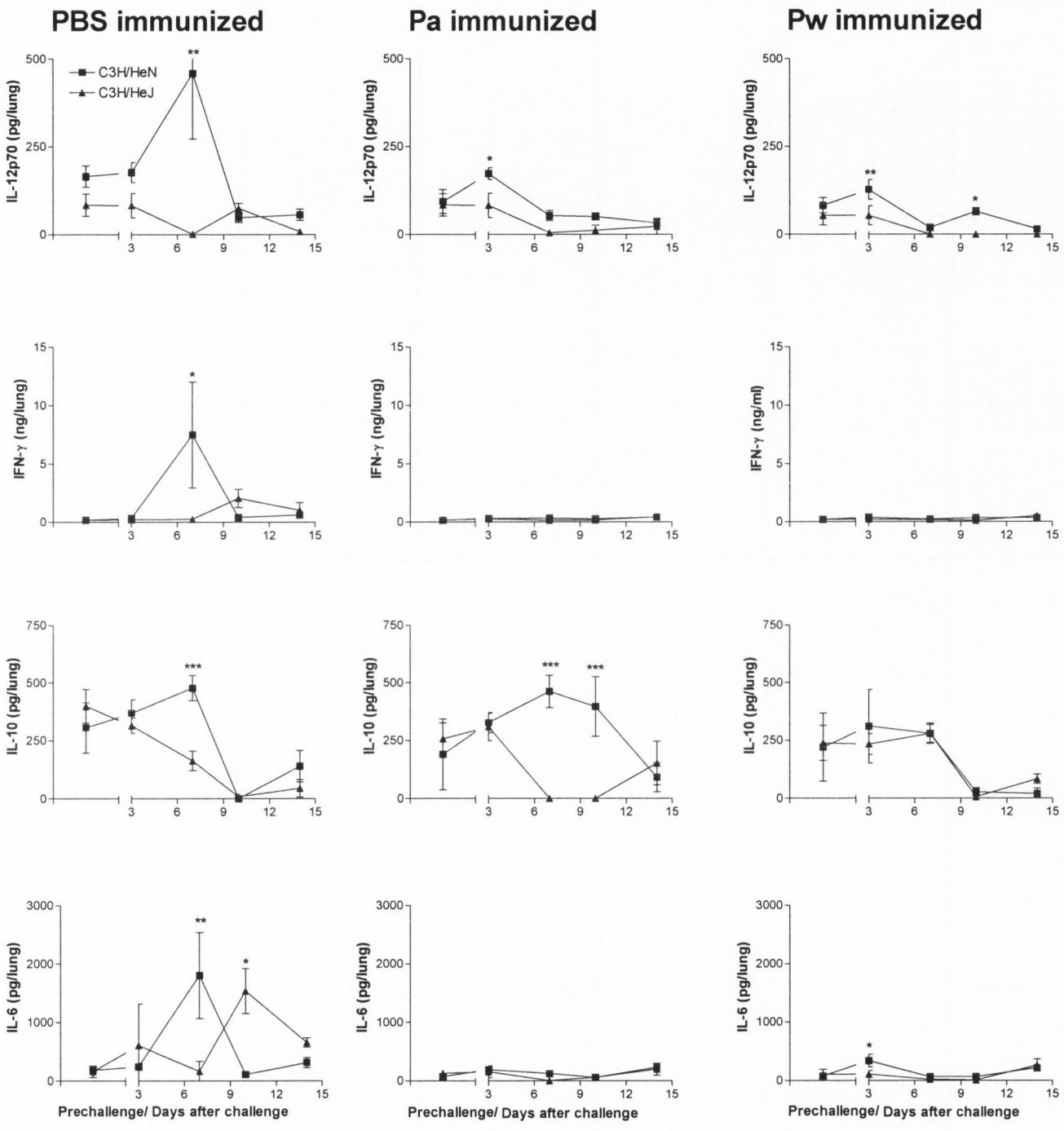

PBS
Pa
Pw
C3H/HeN
C3H/HeJ
CFU Log10 per Lung
7.5
5.0
2.5
0.0
0
3
6
9
12
15
***
*
Days post challenge

Figure 4.2: Differential cytokine and chemokine production in the lungs of C3H/HeN and C3H/HeJ mice immunised with PBS, Pa or Pw prior to and following infection with *B. pertussis*. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at 0 and 4 weeks with $1/5^{th}$ of human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization, mice were aerosol challenged with *B. pertussis*. Cytokine and chemokine analysis were quantified by ELISA on lung homogenates from C3H/HeN and C3H/HeJ mice before challenge or 3, 7, 10, or 14 days after challenge. Results are mean (± SD) for 5 mice per group at each time point. *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

(A)


PBS immunized
Pa immunized
Pw immunized
C3H/HeN
C3H/HeJ
IL-12p70 (pg/lung)
IFN-γ (ng/lung)
IFN-γ (ng/ml)
IL-10 (pg/lung)
IL-6 (pg/lung)
Prechallenge/ Days after challenge

(B)

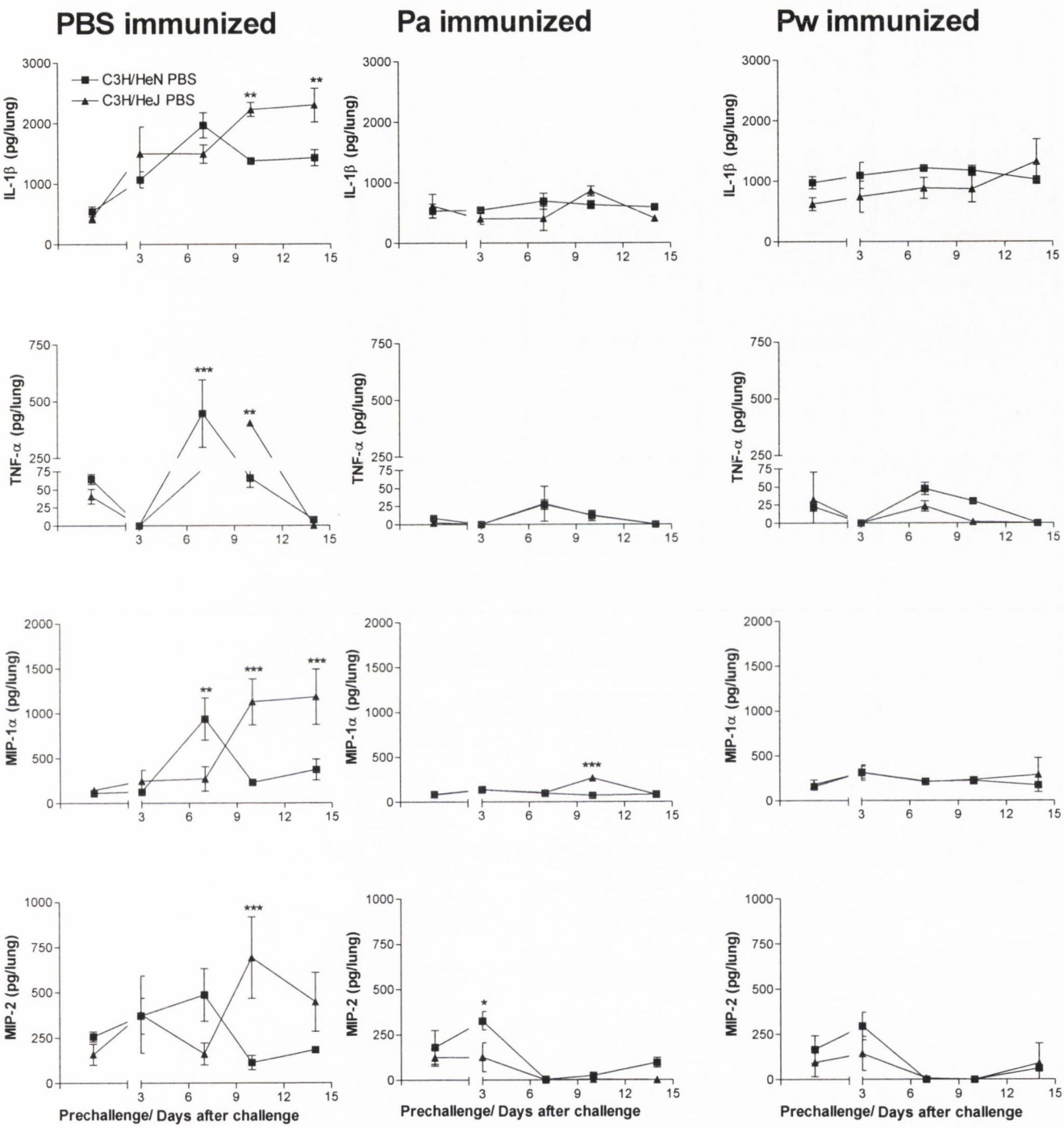

PBS immunized
Pa immunized
Pw immunized
C3H/HeN PBS
C3H/HeJ PBS
IL-1β (pg/lung)
TNF-α (pg/lung)
MIP-1α (pg/lung)
MIP-2 (pg/lung)
Prechallenge/ Days after challenge

Figure 4.3 Reduced *B. pertussis*-specific T cell proliferation responses in TLR4-defective mice immunized with Pa or Pw. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at 0 and 4 weeks with $1/5^{th}$ of human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization spleen cells were isolated from immunized mice and restimulated *in vitro* with *B. pertussis* antigens (FHA 2 μg/ml or formalin-treated *B. pertussis* sonicate (FSON) (1 μg/ml) and PMA/CD3 and medium alone as positive and negative controls respectively. Supernatants were collected 72h later, fresh medium was added to the wells and cells were incubated overnight. At day 4, 2 μCi of ^{3}H thymidine was added to each well. Plates were incubated for a further 6 h, after which cells and ^{3}H incorporation was determined. Results are the mean responses (± SD) for 5 mice assessed individually in triplicate. *, $p< 0.05$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

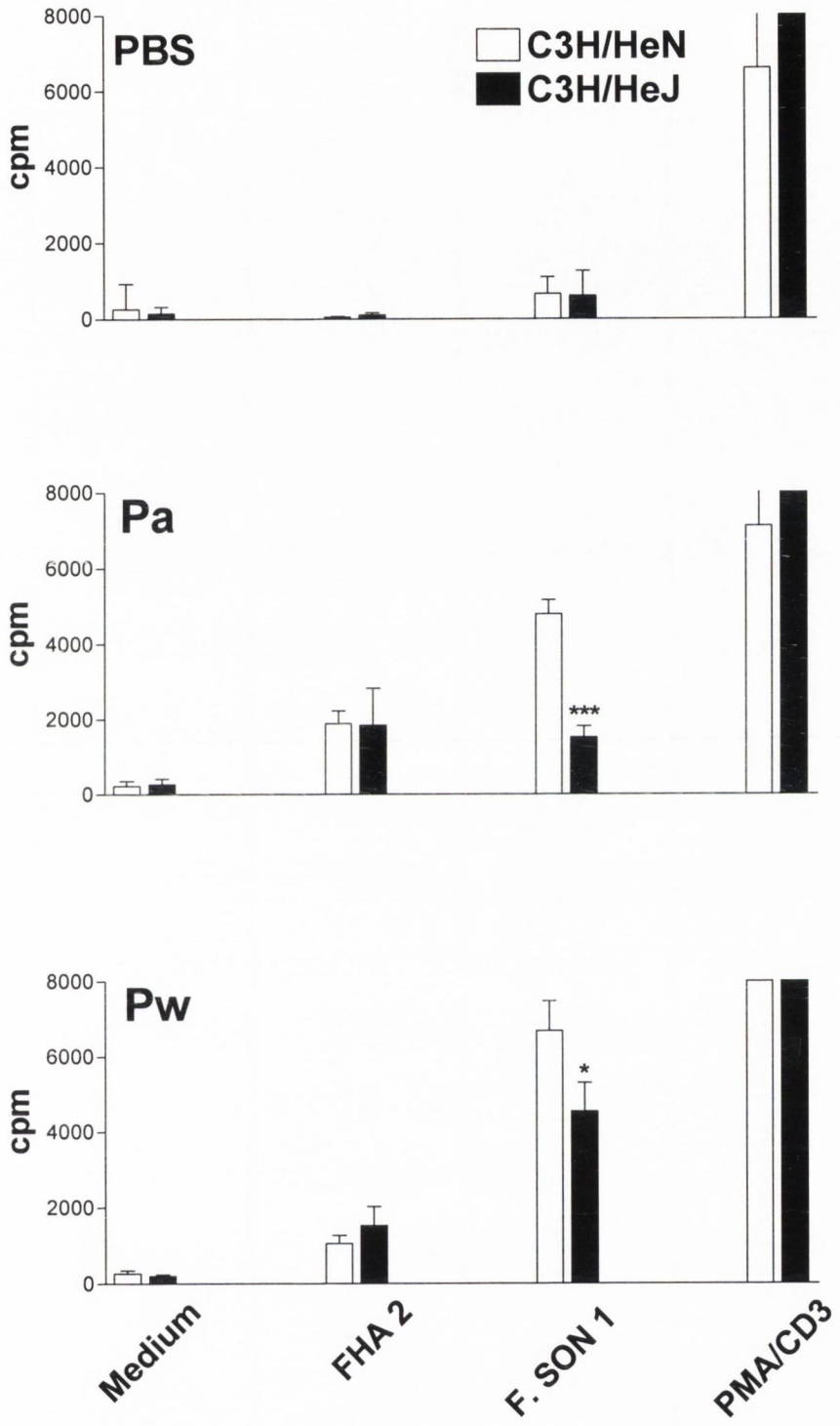

PBS
C3H/HeN
C3H/HeJ
cpm
8000
6000
4000
2000
0
Pa
***
Pw
*
Medium
FHA 2
F. SON 1
PMA/CD3

Figure 4.4: Reduced antigen-specific IL-10 production by spleen cells from C3H/HeJ mice immunized with Pa or Pw. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at 0 and 4 weeks with $1/5^{th}$ of human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization spleen cells were isolated from immunized mice and restimulated *in vitro* with *B. pertussis* antigens (FHA 2 μg/ml or formalin-treated *B. pertussis* sonicate (FSON) 1 μg/ml) and PMA/CD3 and medium alone as positive and negative controls respectively. Supernatants were collected 72h later and concentrations of IFN-γ, IL-10, IL-5 and IL-4 were determined by ELISA. Results are the mean responses (± SD) for 5 mice assessed individually in triplicate. *, $p< 0.05$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.

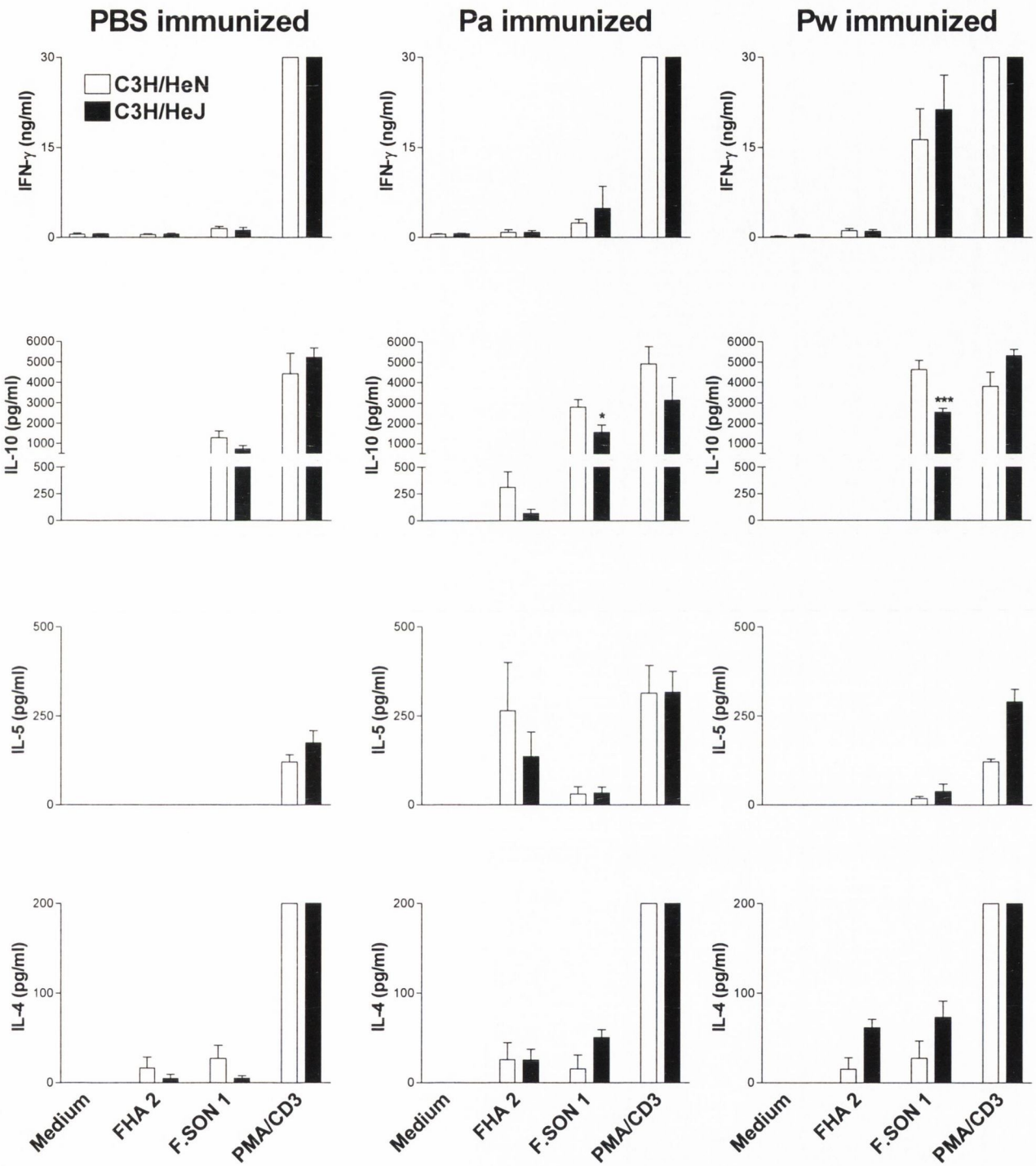
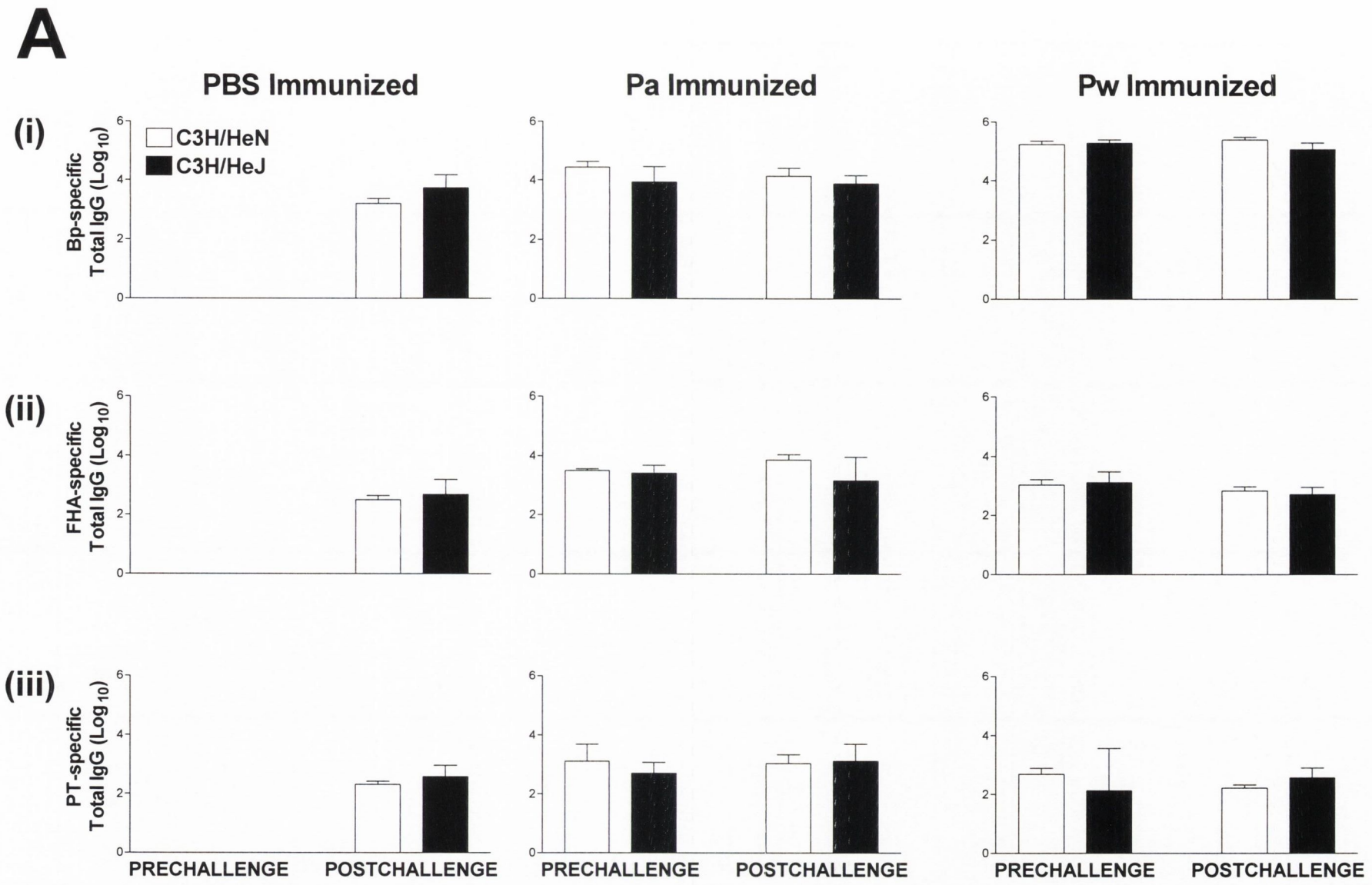
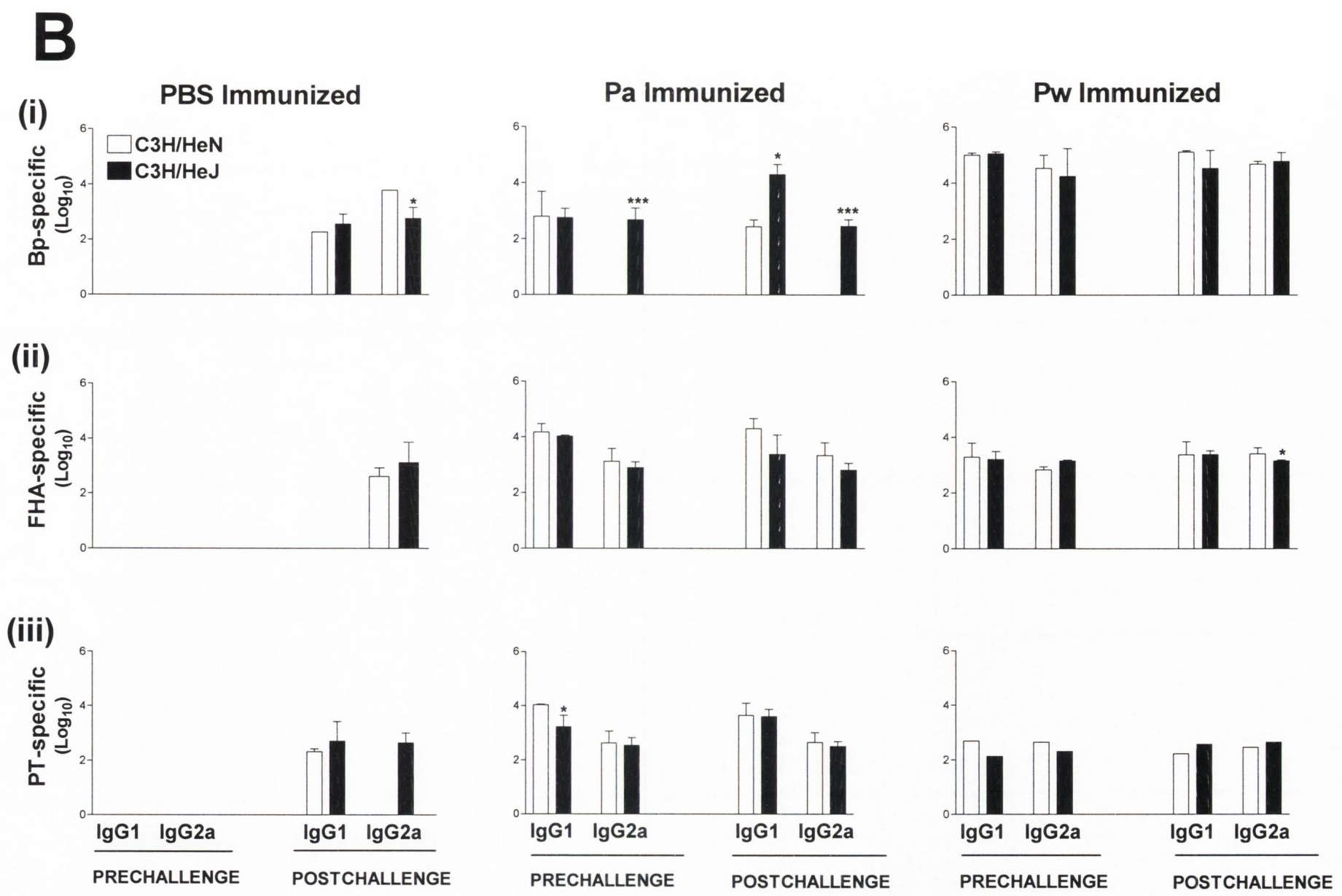

PBS immunized
Pa immunized
Pw immunized
C3H/HeN
C3H/HeJ
IFN-γ (ng/ml)
IL-10 (pg/ml)
IL-5 (pg/ml)
IL-4 (pg/ml)
*
***
Medium
FHA 2
F.SON 1
PMA/CD3

Figure 4.5 Similar *B. pertussis*-specific antibody responses in C3H/HeN and C3H/HeJ mice immunized with Pa or Pw. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at 0 and 4 weeks with $1/5^{th}$ of human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization, mice were aerosol challenged with *B. pertussis* and groups of five mice were sacrificed before and 3h, or 3, 7, 10, or 14 days after challenge. Antibody responses to *B. pertussis* antigens (sonicated *B. pertussis*; Bp, 5 μg/ml; FHA, 2 μg/ml and PT 2 μg/ml) were evaluated by ELISA. Results (Fig 4.5A antigen-specific total IgG titres / Fig 4.5B antigen-specific IgG1 and IgG2a titres) are expressed as mean (± SD) antibody titres for 5 mice per group. *, $p< 0.05$; ***, $p < 0.001$ C3H/HeN vs C3H/HeJ.


A
PBS Immunized
Pa Immunized
Pw Immunized
(i)
(ii)
(iii)
C3H/HeN
C3H/HeJ
Bp-specific Total IgG (Log10)
FHA-specific Total IgG (Log10)
PT-specific Total IgG (Log10)
0
2
4
6
PRECHALLENGE
POSTCHALLENGE


B
PBS Immunized
Pa Immunized
Pw Immunized
(i)
(ii)
(iii)
C3H/HeN
C3H/HeJ
Bp-specific (Log10)
FHA-specific (Log10)
PT-specific (Log10)
0
2
4
6
*
***
IgG1
IgG2a
PRECHALLENGE
POSTCHALLENGE

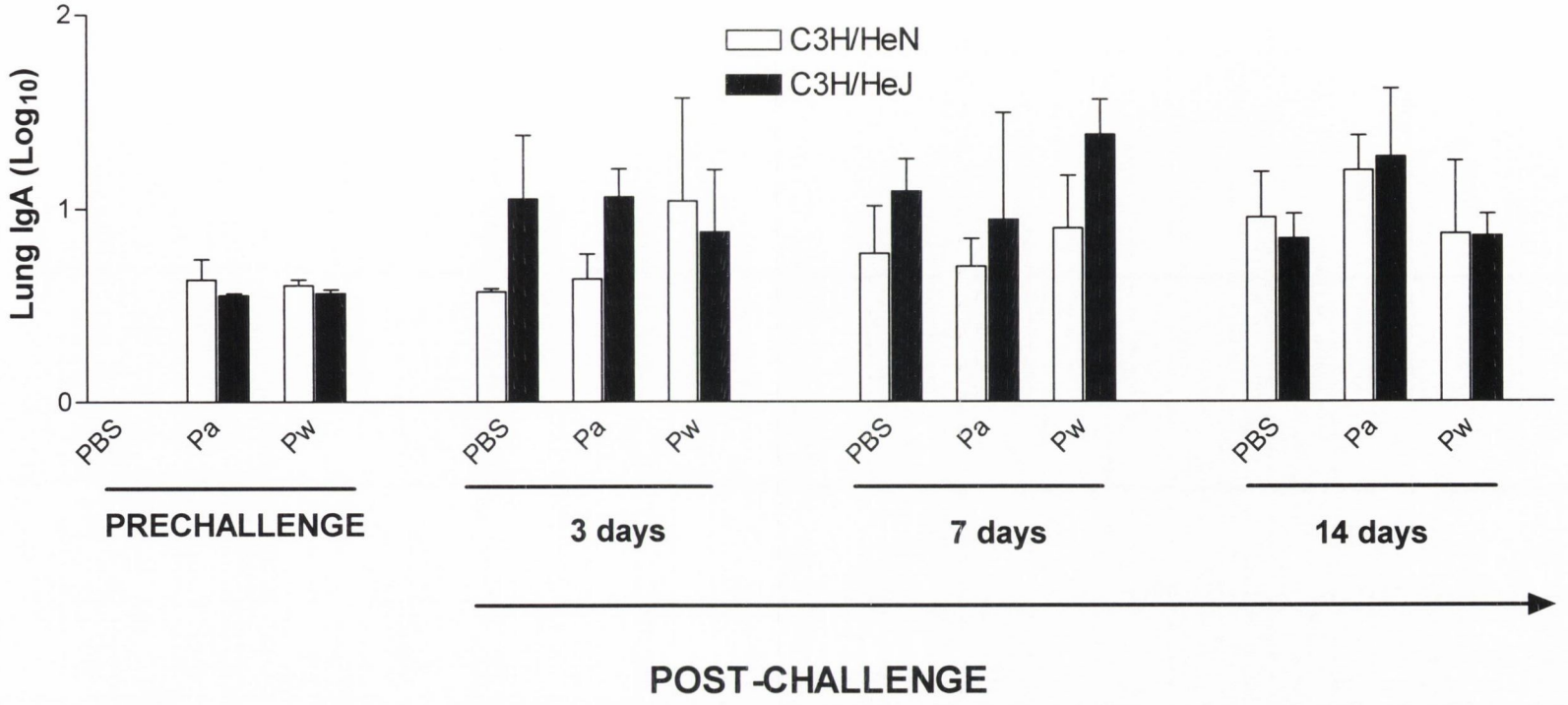
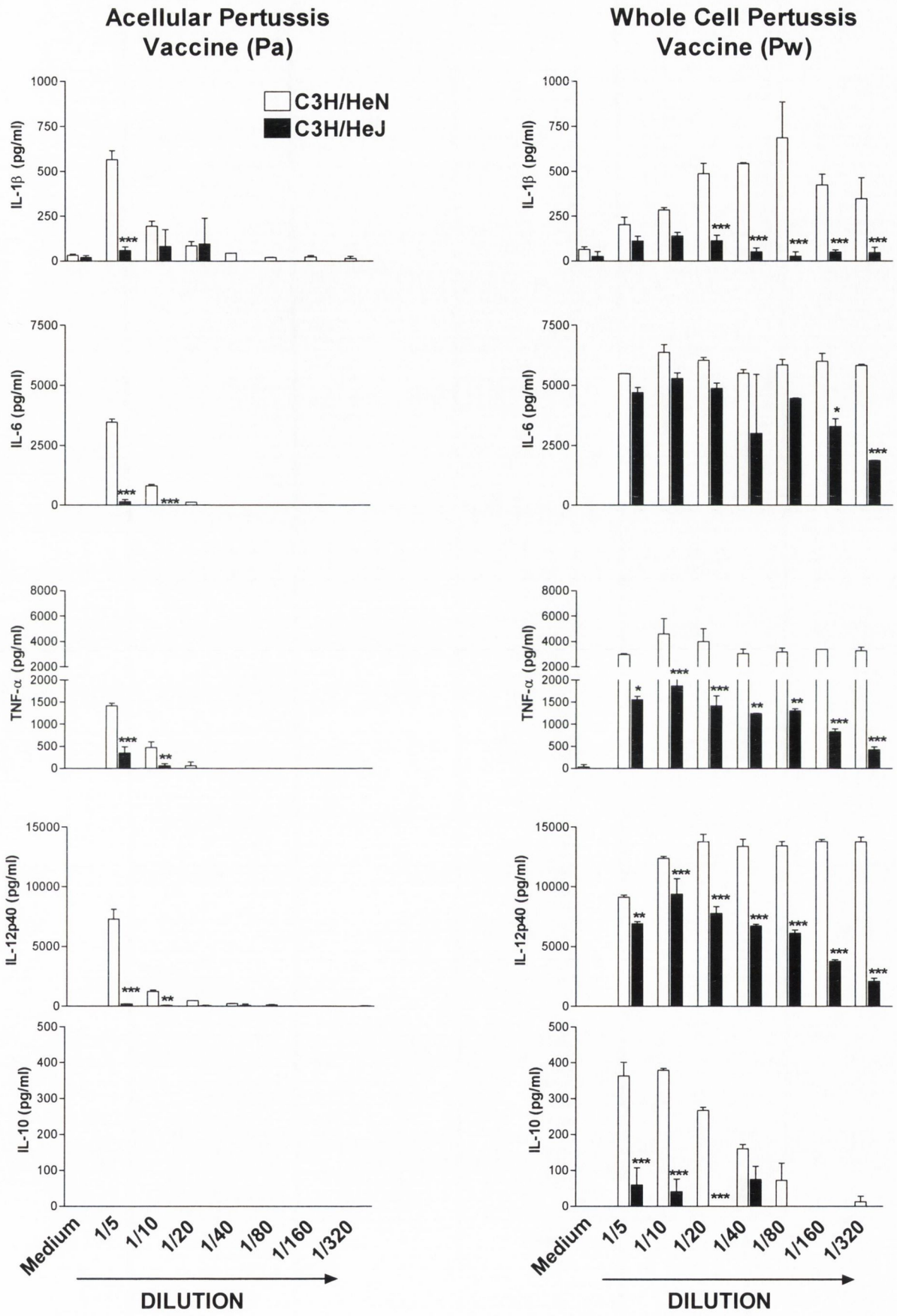


Figure 4.6: Similar IgA production in the lungs of C3H/HeN and C3H/HeJ mice immunized with Pa or Pw. C3H/HeN and C3H/HeJ mice were immunized intraperitoneally at week 0 and 4 with $1/5^{th}$ of a human dose of Pa, Pw or with PBS alone. Two weeks after the second immunization, C3H/HeN and C3H/HeJ mice were aerosol challenged with *B. pertussis* and groups of five mice were sacrificed before or 3, 7, 10, or 14 days later. Lungs were removed and IgA responses to sonicated *B. pertussis* (5 μg/ml) were evaluated by ELISA. Results are expressed as mean antibody titres (± SD) for five mice per group.

Figure 4.7 Components of both Pa and Pw induce pro- and anti-inflammatory cytokine production by signalling through TLR4. Bone-marrow-derived DCs from C3H/HeN and the TLR4-defective C3H/HeJ mice were stimulated with a range of dilutions of Pa (1/5 dilution was equivalent to 0.53 IU) and Pw (1/5 dilution was equivalent to 0.6 IU) Cytokine concentrations were evaluated by ELISA 18 h later. Values represent means ± SD of triplicate assays. $*P<0.05$, $**P<0.01$, ***P< 0.001 versus C3H/HeN.


Acellular Pertussis Vaccine (Pa)
Whole Cell Pertussis Vaccine (Pw)
C3H/HeN
C3H/HeJ
IL-1β (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
IL-12p40 (pg/ml)
IL-10 (pg/ml)
Medium
1/5
1/10
1/20
1/40
1/80
1/160
1/320
DILUTION

Chapter 5

Immunomodulatory properties of Bordetella pertussis Tracheal cytotoxin

5.1 INTRODUCTION

One of the most prominent features of pathology during infection with *B. pertussis* in both animals and humans is the destruction of the ciliated epithelial cell population from the respiratory mucosa. In 1982, Goldman and co-workers reported that a low molecular weight fraction of *B. pertussis* culture could duplicate this pathology when added to hamster tracheal organ cultures (Goldman *et al.*, 1982). The active component in the culture was identified as a 921-Da molecule, called tracheal cytotoxin (TCT). The primary structure of TCT is N-acetylglucosaminyl-1, 6-anhydro-N-acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine (Cookson *et al.*, 1989b). The incorporation of muramic acid and diaminopimelic acid residues occurs in only one molecule in nature, peptidoglycan, which provides structural rigidity to the bacterial cell wall (Schleifer and Kandler, 1972). The structure of TCT places it in the muramyl peptide family, a group of structurally related molecules that are responsible for a diverse range of biological activities. Interestingly, the TCT molecule is identical in structure to the neurologically active slow-wave sleep-promoting factor Fsu, isolated from humans (Cookson *et al.*, 1989a; Martin *et al.*, 1984). In general, muramyl peptides have been shown to have adjuvant, somnogenic, arthritogenic, and pyrogenic activities, which appear to share a common mediator, IL-1 (Krueger, 1990).

Neisseria gonorrhoeae, which also damages human ciliated cells during gonococcal infection of fallopian tube mucosa, releases a 921-Da molecule that is chemically identical to TCT (Sinha and Rosenthal, 1980). More recently, TCT was isolated from the luminous, gram-negative bacterium, *Vibrio fischeri* (Koropatnick *et al.*, 2004). However, the release of TCT is not a general property of gram-negative

bacteria, despite the fact they share a common peptidoglycan composition (Rosenthal *et al.*, 1987).

B. pertussis TCT causes ciliostasis, ciliated cell destruction within cultured hamster respiratory epithelia and can also inhibit DNA synthesis in isolated cultured hamster tracheal epithelial (HTE) cells (Cookson *et al.*, 1989a). TCT inhibition of HTE proliferation may reflect a secondary effect of TCT on the capacity of respiratory epithelium to regenerate the lost ciliated cell population (Cookson *et al.*, 1989a).

IL-1 has been reported to be involved in TCT toxicity in pertussis (Heiss *et al.*, 1993a). Recombinant IL-1 causes TCT-like damage to the respiratory epithelium (Heiss *et al.*, 1993a). IL-1 inhibits DNA synthesis by HTE cells and generates *B. pertussis*-like destruction of epithelial cells in hamster tracheal organ culture (Heiss *et al.*, 1993a). Furthermore, TCT stimulates IL-1α production by respiratory epithelial cells. The IL-1 produced remains intracellular, consistent with the observations that the effects of TCT cannot be blocked using either anti-IL-1α antibodies or the IL-1 receptor antagonist (Heiss *et al.*, 1993a). The ability of exogenously added recombinant IL-1 to duplicate the effects of TCT effects is consistent with an intracellular function for IL-1, since exogenous IL-1 has been shown to be translocated to the nucleus (Mizel *et al.*, 1987). The toxicity of TCT has been linked to the induction of NOS in response to the production of intracellular IL-1 in the respiratory epithelium (Heiss *et al.*, 1994). TCT and endotoxin have also been found to be highly synergistic in the induction of IL-1a, type II iNOS, NO and inhibition of DNA synthesis when added to HTE cells (Flak *et al.*, 2000).

Infiltration of neutrophils, which phagocytose and kill invading microorganisms, represents an important defence mechanism against infection. It has been demonstrated that TCT is toxic for human neutrophils (Cundell *et al.*, 1994). TCT may therefore contribute to the survival of *B. pertussis* within the airways *in vivo*.

Recently, TCT was shown to activate an ancient innate defence pathway in the fruit fly, *Drosophila melanogaster*. Insects depend solely on innate immune responses to survive infection. In *Drosophila*, the IMD pathway (named after 'immune deficient' mutants) is required for antimicrobial gene expression in response to gram-negative bacteria (Lemaitre *et al.*, 1995). Kaneko and co-workers demonstrated that highly purified LPS did not stimulate this pathway. However, the IMD pathway was very sensitive to TCT (monomeric) and polymeric gram-negative peptidoglycans (Kaneko *et al.*, 2004). Activation of the IMD pathway was found to require the diaminopimelic acid residue of gram-negative peptidoglycans.

The present study set out to examine the ability of TCT to stimulate murine bone marrow-derived DC and to investigate if TCT could modulate the response of DC to TLR ligands. This chapter also explored the ability of TCT to function as an adjuvant and to modulate the adaptive immune response to unrelated adjuvants *in vivo*. The findings of this study showed that TCT synergises with TLR2, TLR4 and TLR9 ligands to promote IL-1β production by DC. TCT also down regulated LPS-induced MHC class II expression on DC. Furthermore, TCT exhibited weak adjuvant properties *in vivo* but was capable of impairing the immune response in mice immunised with foreign antigens delivered with powerful adjuvants. These inhibitory effects on T cell responses *in vivo* were mediated through modulatory effects of TCT on DC. Together, these data indicate that the interaction between TCT and the DC

may represent an immune evasion strategy by which *B. pertussis* attempts to subvert protective immune responses.

5. 2 RESULTS

5.2.1 Pre-treatment of BMDC with TCT significantly enhances IL-1β production by TLR2, TLR4 and TLR9 ligands.

Bone marrow-derived DC from BALB/c mice were pre-treated with TCT (10 μg/ml) for 1, 6 and 12 h before being stimulated with a range of concentrations of the TLR2 ligand, Pam-3Csk (10, 100, 1000 ng/ml), a TLR4 ligand, *B. pertussis* LPS (100, 1000, 10,000 pg/ml) and a TLR9 ligand, CpG (10, 100, 1000 ng/ml) for a further 12 h. The concentration range chosen for each TLR agonist was based on preliminary experiments. Treatment of DC with TCT alone did not induce the production of any of of the cytokines and chemokines examined (Fig. 5.1, 5.2, 5.3). However, pre-treatment of DC with TCT (particularly a 1 h pre-treatment) significantly enhanced the production of IL-1β by DC in response to all the TLR ligands examined. A significant enhancement of IL-10 production in response to *B. pertussis* LPS (10, 000 ng/ml) was also detected in DC pre-treated with TCT for 1 and 6 h ($p > 0.05$, $p > 0.001$) (Fig. 5.1). A 12 h pre-treatment of DC with TCT also significantly enhanced *B. pertussis* LPS induced MIP-1α production ($p > 0.01$) (Fig. 5.1).

Pre-treatment of DC with TCT for 1 and 6 h significantly enhanced IL-12p70 production by CpG. Both IL-12p40 and IL-10 production by DC stimulated with CpG were also significantly augmented ($p > 0.001$) after a 1 h pre-treatment with TCT (Fig. 5.2). Although, a 1 h pre-treatment of DC with TCT significantly reduced IL-12p40 production induced by the highest concentration of Pam-3Csk used (1000 ng/ml), a significant increase in IL-12p40 production was detected from DC pre-

treated with TCT for 1 h and stimulated with a lower concentration (100 ng/ml) of Pam-3Csk (Fig 5.3). The synergy observed between TCT and LPS, CpG and Pam-3Csk for IL-12 production suggests that TCT treated DC may enhance Th1-type responses generated with TLR ligands. TLR agonists induced high concentrations of TNF-α production by DC (500 – 5000 pg/ml), which were not affected by pre-treatment with TCT.

Overall, these results indicate that TCT interacts with DC and modulates DC cytokine and chemokine production in response to TLR ligands.

5.2.2 TCT modulates LPS induced MHC Class II expression on DC.

Having demonstrated that TCT modulated the production of cytokines by LPS-, CpG- and Pam-3Csk-stimulated DC, this study next examined the capacity of TCT to modulate the maturation of murine DC. Bone marrow-derived DC from BALB/c mice were cultured for 6 h with TCT (10 μg/ml), stimulated with *B. pertussis* LPS (10 ng/ml, 100 ng/ml) for a further 12 h and then analysed for surface expression of MHC Class II and costimulatory molecules by immunofluorescence analysis with Abs specific for MHC Class II, ICAM-1, CD86 (B7-2) and B7-H1.

Stimulation of DC with TCT (10 μg/ml) did not influence MHC Class II or co-stimulatory molecule expression on DC (Fig 5.4A). In contrast, *B. pertussis* LPS enhanced expression of the DC surface markers examined. MHC Class II expression induced with 10 or 100 ng/ml of *B. pertussis* LPS examined was reduced when DC were pre-treated for 6 h with TCT (Fig. 5.4). TCT did not modulate *B. pertussis* LPS induced expression of the other costimulatory molecules examined. These results

indicate that TCT may be capable of interfering with the presentation of antigen by DC to MHC class II-restricted T cells.

5.2.3 TCT has weak adjuvant activity.

TCT was examined for its ability to function as an adjuvant *in vivo*. BALB/c mice were immunised s.c. into the footpad with KLH (20 μg/mouse) alone or with TCT (10 μg/mouse) and the cellular immune responses were assessed in spleen and local popliteal lymph node cells 7 d later.

First, cell recruitment into draining lymph nodes was evaluated following s.c. injection of KLH or KLH and TCT. The popliteal lymph nodes were removed 7 d after injection and the cell number assessed (Fig. 5.5). A significant increase in cell number was observed in the lymph nodes of mice injected with KLH and TCT ($p < 0.01$) compared with mice immunised with KLH alone (Fig. 5.5).

Spleen or lymph node cells from mice immunised with KLH alone proliferated poorly following antigen stimulation *ex vivo* (Fig. 5.6). Enhanced antigen-specific T cell proliferation responses were detected in spleen cells from mice immunised with TCT and KLH (Fig. 5.6A). However, comparable T cell proliferation responses were detected in lymph node cells from mice immunised with KLH or with KLH and TCT (Fig. 5.6B).

Antigen-specific T cell responses were assessed by stimulating spleen and lymph node cells with KLH *ex vivo*. Spleen (Fig. 5.7) and lymph node cells (Fig. 5.8) from mice immunised with KLH alone produced very low concentrations of cytokines following antigen stimulation *ex vivo*. Similar concentrations of KLH-specific IFN-γ, IL-10, IL-4 and IL-5 were produced by spleen cells from mice immunised with KLH

or KLH with TCT (Fig. 5.7). Likewise, similar concentrations of KLH-specific IFN-γ, IL-10 and IL-4 were detected in the supernatant of popliteal lymph node cells from mice immunised with KLH or KLH and TCT (Fig. 5.8). Although, significant enhancement of antigen-specific IL-5 was detected from lymph node cells from TCT and KLH immunised mice restimulated with 2 μg/ml KLH enhancement of IL-5 was not detected in response to higher concentrations of KLH *ex vivo* (Fig. 5.7).

TCT significantly enhanced ($p > 0.05$) antigen-specific IgG antibody responses following s.c. delivery of KLH (Fig. 5.9). Injection of TCT and KLH induced a slight enhancement of IgG1 and IgG2a subclass responses compared with s.c. delivery of KLH alone.

Overall, TCT did not induce strong antigen-specific cytokine production from spleen and lymph node cells, but did enhance antigen-specific antibody production with a bias towards IgG1, and is capable of recruiting cells to the local draining lymph nodes.

5.2.4 TCT impairs antigen-specific T cell responses elicited by immunisation with antigen adsorbed to alum.

To investigate if TCT could modulate the induction of the immune response to an antigen in the presence of another adjuvant *in vivo*, cohorts of BALB/c mice were injected s.c. into the flank with PBS or TCT alone, and then immunised s.c. with KLH or KLH with alum 6 h later. The adaptive immune response was assessed 14 d later by stimulating spleen and local inguinal lymph node cells with antigen *ex vivo*.

A reduction (although not significant) in the proliferation response to KLH was observed in spleen and lymph node cells from mice pre-injected with TCT 6 h

before immunisation with alum adsorbed KLH (Fig. 5.10). TCT also reduced proliferation response in spleen cells from mice immunised with KLH and PBS.

Alum is a widely used clinical adjuvant, that promotes the induction of Th2 cells. IL-10, IL-4 and IL-5 production, indicative of a Th2 response, was detected from antigen-restimulated spleen cells from mice immunised with KLH and alum (Fig. 5.11). Treatment with TCT 6 h prior to immunisation with KLH and alum resulted in a significant reduction in KLH-specific IL-10 production ($p< 0.001$) by spleen cells. IFN-γ, IL-4 and IL-5 production was also reduced, though not significantly from spleen cells from mice pre-treated with TCT 6 h prior to immunisation with followed by KLH and alum (Fig. 5.11). Notably, the IFN-γ production was reduced in spleen cells from mice treated with TCT before immunisation with KLH and PBS or antigen absorbed to alum (Fig. 5.11). Similar to the response detected in the spleen, KLH-specific IL-10 production by lymph node cells from mice pre-treated with TCT 6 h before immunisation with KLH and alum was severely impaired (Fig. 5.12). Antigen-specific IL-4 and IL-5 production was similar in mice treated with PBS or TCT 6 h before immunisation KLH with alum (Fig. 5.12). A low concentration of antigen-specific IFN-γ was detected in lymph node cells from mice immunised with KLH and alum and this was abrogated in mice pre-treated with TCT.

Injection of TCT 6 h before immunisation with KLH only or KLH and alum had no significant effect on total KLH-specific IgG production or on antigen-specific IgG1 and IgG2a titres (Fig. 5.13).

5.2.5 TCT impairs antigen-specific T cell responses by lymph node cells from mice immunised with antigen and LT as an adjuvant.

Having established a role for TCT in modulating the effects of immunisation with a Th2 promoting adjuvant, alum, an adjuvant that promotes mixed Th1/Th2 responses was employed. To further investigate the ability of TCT to modulate T cell responses *in vivo*, heat labile toxin (LT), produced by some enterotoxigenic strains of *Escherichia coli*, has potent mucosal adjuvant activity and has been used with a wide variety of antigens in animal studies and a number of human clinical trials. The adjuvant effect of LT has been demonstrated in studies involving immunisation via the subcutaneous, intraperitoneal, intravenous, intradermal and transcutaneous routes (Glenn *et al.*, 2000; Scharton-Kersten *et al.*, 2000; Weltzin *et al.*, 2000). This study examined the ability of TCT to modulate the antigen-specific immune responses generated with LT as the adjuvant. Groups of 5 BALB/c mice were injected s.c. into the flank with PBS or TCT (10 μg/mouse) and then immunised with KLH (20 μg/mouse) or KLH with LT (100 ng/mouse) 6 h later. The T cell response was assessed 14 d later from spleen and local inguinal lymph node cells stimulated with antigen *ex vivo*.

Spleen or lymph node cells from mice immunised with KLH alone proliferated very poorly following antigen stimulation *ex vivo* (Fig. 5.14). Enhanced antigen-specific T cell proliferation responses were detected in spleen and lymph node cells from mice immunised with KLH in the presence of LT (Fig. 5.14). T cell proliferation was not altered in spleen and lymph node cells from mice pre-injected with TCT 6 h before immunisation with LT and KLH (Fig. 5.14).

Consistent with previous studies, which have shown that LT, induced a mixed Th1/Th2 type response, antigen-specific IFN-γ, IL-10 and IL-5 production was detected in spleen cells from mice immunised with KLH and LT (Fig. 5.15). No KLH-specific IL-4 production was detected from spleen cells of any of the immunised mice. A decrease (although not significant) in KLH-specific IL-10 production by spleen cells was observed in mice pre-treated with TCT before immunisation with KLH and LT. Pre-injection with TCT did not impair antigen-specific IFN-γ or IL-5 production by spleen cells in mice immunised with KLH with LT (Fig. 5.15).

A mixed Th1/Th2 response was also evident in lymph node cells from mice immunised with LT and KLH, with IFN-γ, IL-10, IL-4 and IL-5 production detected in response to KLH stimulated *ex vivo* (Fig. 5.16). Pre-injection with TCT 6 h before immunisation with KLH and LT significantly impaired the antigen-specific IFN-γ and IL-4 production by the lymph node cells (Fig. 5.16). A reduction in KLH-specific IL-10 and IL-5 production was also evident in lymph node cells from mice injected with TCT 6 h before immunisation KLH with LT.

Injection with TCT 6 h before immunisation with LT and KLH had no effect on total KLH-specific IgG production or on antigen-specific IgG1 and IgG2a titres (Fig. 5.17).

Taken together, these results indicate that injection of TCT 6 h before immunisation with LT and KLH impairs the antigen-specific T cell cytokine response in the local lymph nodes but does not appear to affect T cell proliferation or antibody production.

5.2.6 TCT-treated DC impairs induction of T cell responses *in vivo*.

Standard protocols to generate murine DC generally use culture medium supplemented with FCS; however, *in vivo* transfer of DC cultured in FCS results in priming of T cells to xenogeneic antigens in the FCS that complicate the interpretation of DC adoptive transfer experiments (Muller *et al.*, 2000). To overcome this problem, bone marrow-derived DC from BALB/c mice were cultured in 2 % heat inactivated normal mouse sera and 40 ng/ml recombinant GM-CSF for 12 d. DC were harvested and cultured O/N at a concentration of 1 x 10^6 cells/ml. DC were then incubated with KLH (20 μg/ml) alone, KLH (20 μg/ml) and TCT (10 μg/ml), KLH (20 μg/ml) plus Pam-3Csk (TLR2 ligand; 500 ng/ml), or with KLH (20 μg/ml) and TCT (10 μg/ml) and Pam-3Csk (500 ng/ml). After 2 h incubation, cells were washed and injected s.c into the flank of BALB/c mice (1 x 10^5 cells/mouse). T cell responses were assessed 14 d later from pooled local inguinal lymph node cells stimulated with antigen *ex vivo*.

Lymph node cells were also examined for their ability to proliferate. Lymph node cells from mice that received DC treated with Pam-3Csk with KLH proliferated strongly (Fig. 5.18). There was a profound impairment of KLH-specific proliferation by lymph node cells from mice that received DC treated with TCT and Pam-3Csk with KLH (Fig. 5.18). Furthermore, the moderate proliferation detected by lymph node cells from mice injected with KLH treated DC alone was almost abolished in mice that received DC also treated with TCT.

Lymph node cells from mice transferred with DC treated with KLH and Pam-3Csk produced moderate concentrations of IFN-γ and IL-10 (Fig. 5.19). This antigen-specific IFN-γ and IL-10 production was completely abolished in mice that received

DC also treated with TCT (fig. 5.19). High concentrations of antigen-specific IL-10 and IL-4 were detected from lymph node cells from mice injected with KLH treated DC, production of these cytokines was abolished in mice transferred with DC that had also been treated with TCT. Antigen-specific IL-5 production was not above background concentrations in LN cells from all treatment groups. No KLH-specific IFN-γ, IL-10 and IL-4 production was detected in lymph node cells from mice that received DC treated with TCT and KLH.

Overall, these data demonstrate that the ability of TCT to modulate the induction of an antigen-specific T cell response *in vivo* appears to result, at least in part, from it's effects on DC.

5.3 DISCUSSION

The innate immune system represents the first line of defence against bacterial colonisation and infection (Medzhitov and Janeway, 2000). The innate system defends the host by preventing bacterial adherence to mucosal surfaces (e.g. ciliary movement and mucus production), and the release of antimicrobial factors (e.g. β-defensins). The subsequent immune response to an invading pathogen is also determined by the innate response, through the recognition of specific bacterial molecules (e.g. LPS) using PRRs (Medzhitov and Janeway, 2000). The success of many pathogens lies in their ability to circumvent, resist and counteract such host defence mechanisms (Hornef *et al.*, 2002). *B. pertussis* paralyses the ciliary clearance function of the respiratory tract through the release of a 921-Da peptidoglycan fragment, TCT, a component of the bacterial cell wall. The NO-mediated ciliostasis induced by TCT facilitates the survival and replication of *B. pertussis* in the respiratory tract (Cookson *et al.*, 1989a). Infiltration of neutrophils, which phagocytose and kill invading microorganisms, is an important defence mechanism. It has been reported that TCT is toxic for human neutrophils, which may contribute to the survival of *B. pertussis* in the airways *in vivo* (Cundell *et al.*, 1994). TCT, therefore, appears to make a vital contribution to the evasion strategies of *B. pertussis*.

DC play a crucial role in shaping the host immune response to invading pathogens. The present study focused on TCT and its ability to interact with the DC. The adaptive response induced by TCT and its ability to modulate the host's adaptive response following immunisation with antigens in presence of other adjuvants was also investigated. The significant findings of this study are that TCT has suppressive properties *in vivo* and that this suppression may stem from its interaction with the DC.

It is well established that the adjuvant activity of peptidoglycan is attributed to the muramyl peptide structure, muramyl dipeptide (MDP) (Ellouz *et al.*, 1974). It has previously been reported that TCT and LPS synergise to induce production of IL-1α, type II (inducible) nitric oxide synthase, nitric oxide production and the inhibition of DNA synthesis in hamster tracheal epithelial (HTE) cells (Flak *et al.*, 2000). Others have reported a synergistic effect of other muramyl peptides with LPS. Yang *et al.*, demonstrated that MDP could synergise with LPS or lipoteichoic acid to induce IL-8 production in human monocytic cells (Yang *et al.*, 2001). Wang and co-workers (2001) reported that co-administration of MDP with LPS caused significantly increased concentrations of TNF-α and IL-6 in cultures of whole human blood, whereas IL-10 production was not influenced (Wang *et al.*, 2001). Wolfert *et al.* (2002) reported that MDP synergises with LPS or peptidoglycan to induce synthesis of TNF-α in the human monocytic cell line Mono Mac 6 (Wolfert *et al.*, 2002). Recently, chemically synthesised MDP and several chemically synthesised muramyl peptide derivatives were reported to synergise for TNF-α, IL-1β, IL-6 and IL-10 production from whole human blood cultures (Traub *et al.*, 2004). The authors proposed that different stimulus concentrations, as well as differences in incubation time may explain why a previous study by Wang *et al.* (2001) did not detect synergy for IL-10 production by MDP with LPS. IL-10 production was detected late after the initial activation of monocytes and not released in significant amounts before 8 h (Traub *et al.*, 2004). This observation highlights the importance of using several incubation times in experiments. In the present study, bone marrow-derived DC were pre-treated with TCT for 1, 6, and 12 h before stimulation with various TLR ligands. Consistent with the synergistic interaction reported for TCT with LPS in HTE cells (Flak *et al.*, 2000), TCT synergised with LPS to induce IL-1β production by DC.

Furthermore, synergy between TCT and LPS extended to the production of anti-inflammatory cytokine IL-10 and the chemokine, MIP-1α. Although, many studies using the related molecule MDP observed a synergistic effect with LPS for TNF-α production in human cell lines or human blood (Traub *et al.*, 2004; Wang *et al.*, 2001; Wolfert *et al.*, 2002), TCT did not synergise with LPS for TNF-α production by DC. Until recently, there were no reports of the synergistic effects between MDP and other TLR ligands such as TLR2 or TLR9 ligands. Two types of synthetic peptidoglycan fragments, diaminopimelic acid-containing desmuramyl peptide (DMP) and MDP have been reported to synergise with the TLR2 ligand, Pam-3Csk and with the TLR9 ligand CpG for IL-8 secretion in human monocytic THP-1 cells (Uehara *et al.*, 2005). The present study has reported that TCT synergises with CpG for IL-1β, IL-12p70, IL-12p40 and IL-10 production from murine DC. Similarly, a synergistic interaction was observed between TCT and Pam-3Csk for IL-1β production by DC. TCT therefore, exhibits an ability to synergise with various TLR ligand similar to its structurally related compound, MDP.

Antigen processing and presentation plays a central role in the induction of adaptive immune responses. Following bacterial uptake, antigens from bacteria are processed by professional APC, such as the DC, and degraded peptides are presented in association with MHC molecules to T cells. $CD4^+$ T cells recognise processed antigens in association with MHC class II molecules, which have been processed by an exogenous route of antigen processing (McDevitt, 2000). Recent reports have shown that several types of bacteria, including *mycobacteria, E. coli, Yersinia enterocolitica,* and *B. pertussis* can decrease MHC class II expression on infected cells, inhibiting antigen processing and presentation to support their survival (De Lerma Barbaro *et al.*, 1999; Noss *et al.*, 2000; Noss *et al.*, 2001; Shumilla *et al.*, 2004;

Wojciechowski *et al.*, 1999; Zhong *et al.*, 1999). Inhibition of MHC class II expression and antigen processing by *M. tuberculosis* is mediated by a 19 kDa lipoprotein via a TLR2-dependent mechanism (Noss *et al.*, 2001). More recently, Fulton and colleagues reported the inhibition of MHC class II expression and antigen processing in murine alveolar macrophages by *M. bovis* bacillus-calmette guerin (BCG) and the 19 kDa mycobacterial lipoprotein (Fulton *et al.*, 2004). Suppression of MHC class II expression has also been reported in *E. coli* infected THP-1 cells and human monocytes (De Lerma Barbaro *et al.*, 1999). A marked but transient downregulation of MHC class II expression was reported on human DC 3 days after infection with *Y. enterocolitica* (Schoppet *et al.*, 2000). *B. pertussis* infection of human monocytes reduced cell surface expression of HLA-DR without altering total cellular levels. This change in cell surface HLA-DR reflected intracellular redistribution (Shumilla *et al.*, 2004). Using a PT mutant strain of *B. pertussis*, the authors identified that pertussis toxin partially accounts for the decrease in HLA-DR cell surface expression (Shumilla *et al.*, 2004). The present study illustrated that TCT is capable of inhibiting LPS-induced MHC class II expression on DC. This observation again highlights a role for TCT in the immune evasion strategies of *B. pertussis*. Shumilla and co-workers also reported that *B. pertussis* decreased cell surface expression of CD86 on human monocytes. Bacterial cell wall (BCW) extracts from gram-positive *Eubacterium spp.* and *Lactobacillus spp.* were also recently reported to downregulate the expression of HLA-DR and CD86 on human PMBCs (Chen *et al.*, 2002). TCT did not however, modulate CD86 expression on murine DC (present study).

Since T cell activation is an essential step in the immune response to an infectious pathogen, many bacteria have evolved strategies to evade adaptive

immunity by suppressing T cell responses. The immunosuppressive properties of gram-positive BCW were initially identified in a streptococcal cell wall-induced arthritis model *in vivo* (Regan *et al.*, 1988; Wahl *et al.*, 1988). Rats exhibited a profound immunosuppression (specifically suppressed T cell proliferation) after an i.p injection of streptococcal BCW (Regan *et al.*, 1988; Wahl *et al.*, 1988). A model of *Eubacterium* BCW-induced arthritis also demonstrated T cell suppression (Zhang *et al.*, 2000). Non-specific immunosuppression by other BCW components has also been reported, in both *in vitro* and *in vivo* models (Ellner and Daniel, 1979; Kaplan *et al.*, 1985; Prasad *et al.*, 1987). In severe cases of tuberculosis and lepromatous leprosy, patients suffer from a non-specific unresponsiveness with compromised T cell functions, not only towards the relevant antigen (lepromin in the case of lepromatous leprosy), but also towards other antigens, such as PPD (mycobacterial antigen) (Kaplan *et al.*, 1985). In a study undertaken by Chen *et al.* (2002 gram-positive BCWs from *Eubacterium spp.*, *Lactobacillus spp.*, and the pathogen *Streptococcus pyrogenes* were shown to suppress the proliferation responses of PBMC against several bacterial antigens (PDD and tetanus toxoid) and viral antigens (purified adenovirus hexon protein, virions of poliovirus type 1 and herpes simplex viral antigen) but not against mitogens (Chen *et al.*, 2002). Boschwitz and co-workers reported that after incubation with *B. pertussis*, human monocytes were unable to stimulate tetanus toxoid-specific $CD4^+$ T cells to the same degree as unexposed control cells (Boschwitz *et al.*, 1997). Furthermore, mutant strains deficient in FHA or ACT were incapable of inhibiting proliferation, suggesting that these virulence factors are essential for this activity. In contrast, a PT mutant was not capable of inhibiting tetanus toxoid-specific T cell proliferation (Boschwitz *et al.*, 1997). The present study shows that TCT impairs the T cell response to bystander antigen

delivered with LT or alum as the adjuvant *in vivo*. Antigen-specific IL-10 production was significantly decreased ($p > 0.001$) in spleen cells from mice treated with TCT 6 h before immunisation with alum and KLH. Pre-injection with TCT also resulted in a reduction in antigen-specific IFN-γ, IL-4 and IL-5 in spleen cells from mice immunised with alum and KLH. TCT was also able to strongly reduce the KLH-specific proliferation response in spleen and lymph node cells from mice when pre-treated with TCT before immunisation with KLH adsorbed to alum. Pre-treatment of mice with TCT also suppressed antigen-specific IL-4, IL-5, IFN-γ and IL-10 in response to KLH formulated with LT, an adjuvant that induces mixed Th1/Th2 type responses.

Anthrax lethal toxin, the major virulence factor of *Bacillus anthracis*, exerts profound inhibitory effects on DC, ultimately resulting in an impaired adaptive immune response (Agrawal *et al.*, 2003). Isolated spleen DC, exposed to anthrax protective antigen (PA) alone or with anthrax lethal toxin and OVA were transferred into BALB/c mice reconstituted with DO11.10 cells. The OVA-specific T cell response in draining lymph nodes was impaired in mice that received DC treated with PA and anthrax lethal toxin when compared with mice treated with PA only treated DC (Agrawal *et al.*, 2003). Using a combination of MAP kinase inhibitors, the authors established a role for MAP kinases in anthrax lethal toxin mediated inhibition of DC function (Agrawal *et al.*, 2003). The DC may therefore be a target for the evasion strategies of many bacteria. Like *B. anthracis*, the present study showed that *B. pertussis* may subvert the host's immune response by targeting the DC. Further evidence for this was provided by the observation that treatment with TCT inhibited the ability of Pam-3Csk-activated DC to stimulate antigen-specific IFN-γ and IL-10 following transfer into naïve mice. Furthermore, a profound impairment of KLH-

specific proliferation by lymph node cells was demonstrated in mice transferred with DC pulsed with TCT and Pam-3Csk when compared with transfer of DC pulsed with Pam-3Csk alone. This suggests that TCT mediates its inhibitory effect on T cell responses through a profound inhibitory effect on DC function.

This study has not established the precise mechanism by which TCT acts on DC. Two members of the NOD (nucleotide-binding oligomerization domain) family, NOD1 and NOD2 have recently been implicated in the recognition of specific bacterial components (Inohara and Nunez, 2003). These cytoplasmic proteins are involved in the detection of bacterial molecular structures that enter into the cell (Viala *et al.*, 2004). Purified peptidoglycan from gram-negative bacteria, including *E. coli* and *Shigella flexneri*, has been shown to interact with NOD1, leading to NF-κB activation (Viala *et al.*, 2004). The minimal peptidoglycan fragment that stimulates NOD1 is a disaccharide of N-acetyl glucosamine-N-acetyl muramic acid linked to a tripeptide where the terminal amino acid is meso-diaminopimelic acid (meso-DAP). The presence of DAP in peptidoglycan can be considered as a general signature of gram-negative bacteria, since peptidoglycan from most, but not all, gram-positive bacteria have lysine (Viala *et al.*, 2004). However, the tripeptide structure binds NOD1 with high specificity since the presence of an additional amino acid to the DAP abrogates the binding of this bacterial product to NOD1 (Girardin *et al.*, 2003a). TCT is a tetrapeptide molecule, which suggests that TCT may not bind to NOD1 in DC. NOD2 recognises the MDP moiety of peptidoglycan. Since MDP forms part of both gram-positive and gram-negative peptidoglycan, NOD2 is regarded as a general sensor of bacterial infection (Viala *et al.*, 2004). NOD2 expression is mainly restricted to macrophages but expression can be upregulated in epithelial cells upon treatment with pro-inflammatory stimuli such as IFN-γ and TNF-α (Gutierrez *et al.*,

2002; Rosenstiel *et al.*, 2003). Overall, this suggests that it is unlikely that NOD1 and NOD2 are involved in the recognition of the *B. pertussis* muramyl peptide, TCT. However, further studies are required to discount a role for NOD1/NOD2 in TCT mediated signalling.

RelB, a member of the NF-κB family of transcription factors is essential for DC maturation and antigen presentation of bone marrow-derived DC (Zanetti *et al.*, 2003). A recent report showed that RelB is exclusively repressed by NF-κB2/p100 in HeLa cells (Solan *et al.*, 2002). A report by Speirs *et al.* (2004) showed that RelB is highly active in NF-κB2/p100 knock out (KO) DC. In the absence of NF-κB2 DC are hyperactivated, showing increased MHC class II and costimulatory molecule expression, both constitutively and in response to stimuli (Speirs *et al.*, 2004). NF-κB2 KO DC were also shown to be more efficient (up to 10 times) than wildtype DC in inducing activation of $CD4^+$ T cells. The authors concluded that NF-κB2 was a critical regulator of DC function (Speirs *et al.*, 2004). Perhaps TCT functions to prevent the dissociation of NF-κB2/p100 from RelB in DC in response to a stimuli such as LPS or Pam-3Csk. Repressed RelB activity would result in decreased ability to induce $CD4^+$ T cell response. This is however, only one of the many possibilities. Similar to anthrax lethal toxin, TCT may target the MAP kinase pathway in DC. Alternatively, TCT may sequester MHC Class II molecules intracellularly. The signalling pathways activated by TCT in DC need to be defined in order to fully appreciate the immune evasion strategy deployed by this peptidoglycan fragment.

Caspases are a large family of serine proteases and use cysteine as the nucleophilic group to cleave substrate at the C terminus of aspartic acid (Los *et al.*, 2001). Caspases have been extensively characterised in the context of their function

in apoptosis (Los *et al.*, 2001). However, mammalian caspases have also evolved additional roles in the inflammatory response (Los *et al.*, 2001). More recently, caspases have been implicated in T cell activation (Alam *et al.*, 1999; Chun *et al.*, 2002). Recently, a study by Wong and co-workers (2004) have demonstrated an additional role for caspases in the regulation of endosomal trafficking pathways that appears to include MHC class II distribution during maturation of DC (Wong *et al.*, 2004). In immature bone marrow derived-DC, a number of molecules involved in intracellular trafficking (e.g. α-adaptin (AP-1 complex) and γ-adaptin (AP-2 complex)) were present in cleaved form, degraded by caspase-like proteases. Cleavage was either inhibited or significantly reduced during maturation of DC induced by LPS by peptides that inhibit caspase activity (caspase –1, -3, -4 –7 and –6, -8, -9, -10). Furthermore, treatment of DC with LPS or caspase inhibitor resulted in the expression of MHC class II on the DC surface. The authors concluded that changes in cell surface expression of MHC class II are regulated at least in part by the activities of caspases, inducible NO, and its product NO (Wong *et al.*, 2004). A study investigating caspase activity in DC stimulated with TCT may yield an important insight into how TCT interferes with DC activation of T cells.

In conclusion, this study has established that TCT interacts with murine DC, which potentiate certain innate responses to other TLR ligands *in vitro*, especially IL-1β and IL-10 production. However, incubation of TCT with DC also suppresses their ability to promote T cell responses *in vivo*. The immunomodulatory properties of TCT *in vivo* were illustrated when administered with bystander antigen formulated with other adjuvants. Further study is required to elucidate the mechanism that TCT employs to modulate the APC function of the DC. Such knowledge will contribute to our understanding of *B. pertussis* pathogenesis.

Figure 5.1 Pretreatment of BMDC with TCT enhances IL-1β production in response to the TLR-4 ligand, *B. pertussis* LPS. Bone marrow-derived DC from BALB/c mice were pretreated with TCT (10 μg/ml) for 1, 6 and 12 h before further stimulation with *B. pertussis* LPS (Bp LPS; 100, 1000, 10000 pg/ml) for 12 h. Cytokine and chemokine concentrations were evaluated by ELISA. Values represent means ± SD of triplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ TCT-treated DC versus un-treated DC

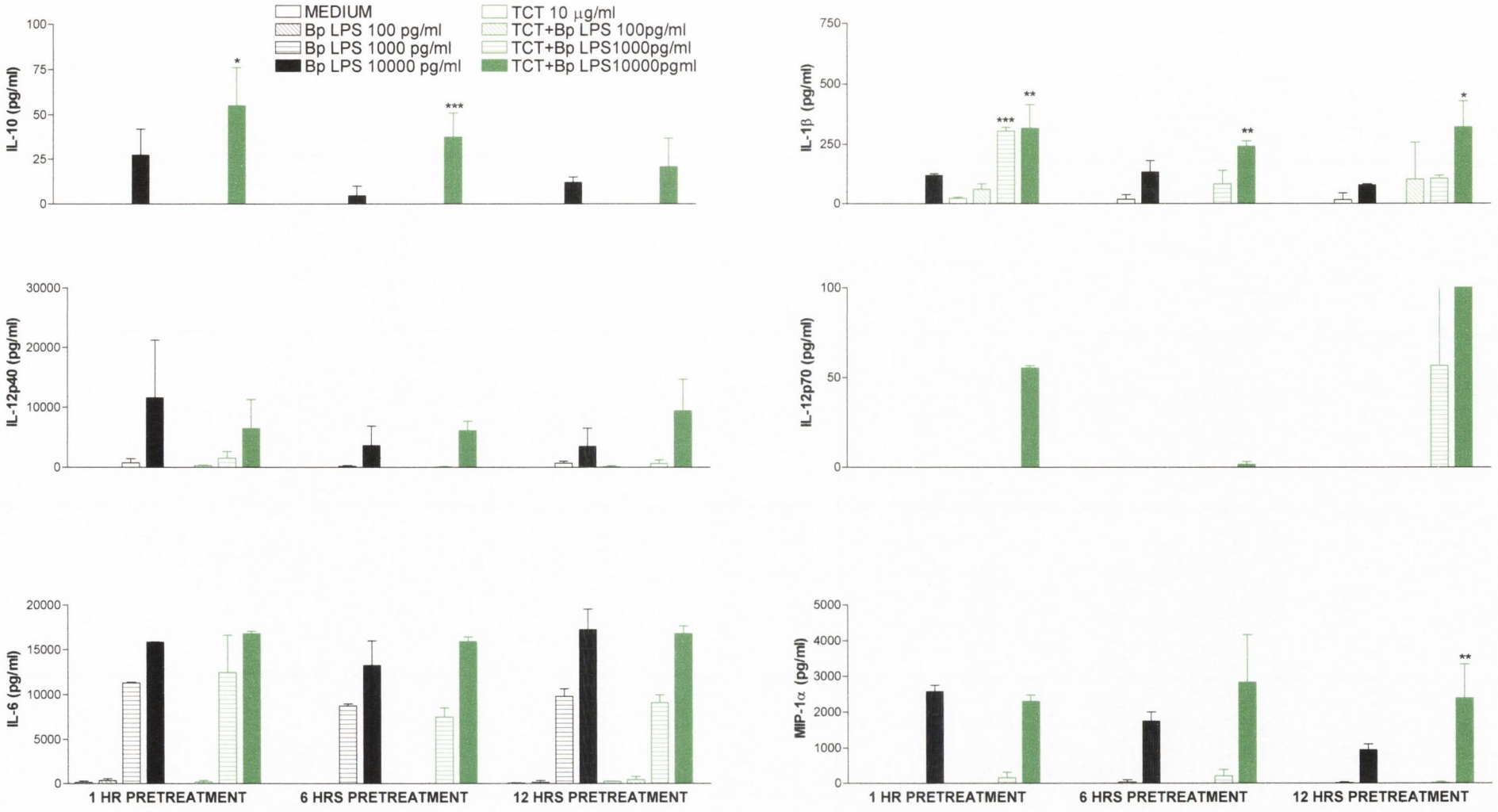

MEDIUM
Bp LPS 100 pg/ml
Bp LPS 1000 pg/ml
Bp LPS 10000 pg/ml
TCT 10 μg/ml
TCT+Bp LPS 100pg/ml
TCT+Bp LPS1000pg/ml
TCT+Bp LPS10000pgml
IL-10 (pg/ml)
IL-1β (pg/ml)
IL-12p40 (pg/ml)
IL-12p70 (pg/ml)
IL-6 (pg/ml)
MIP-1α (pg/ml)
1 HR PRETREATMENT
6 HRS PRETREATMENT
12 HRS PRETREATMENT

Figure 5.2 Pretreatment of BMDC with TCT enhances the production of IL-1β,IL-12 and IL-10 in response to the TLR-9 Ligand, CpG. Bone marrow-derived DC from BALB/c mice were pretreated with TCT (10 μg/ml) for 1, 6 and 12 h before further stimulation with CpG (10, 100, 1000 ng/ml) for 12 h. Cytokine and chemokine concentrations were evaluated by ELISA. Values represent means ± SD of triplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ TCT-treated DC versus un-treated DC

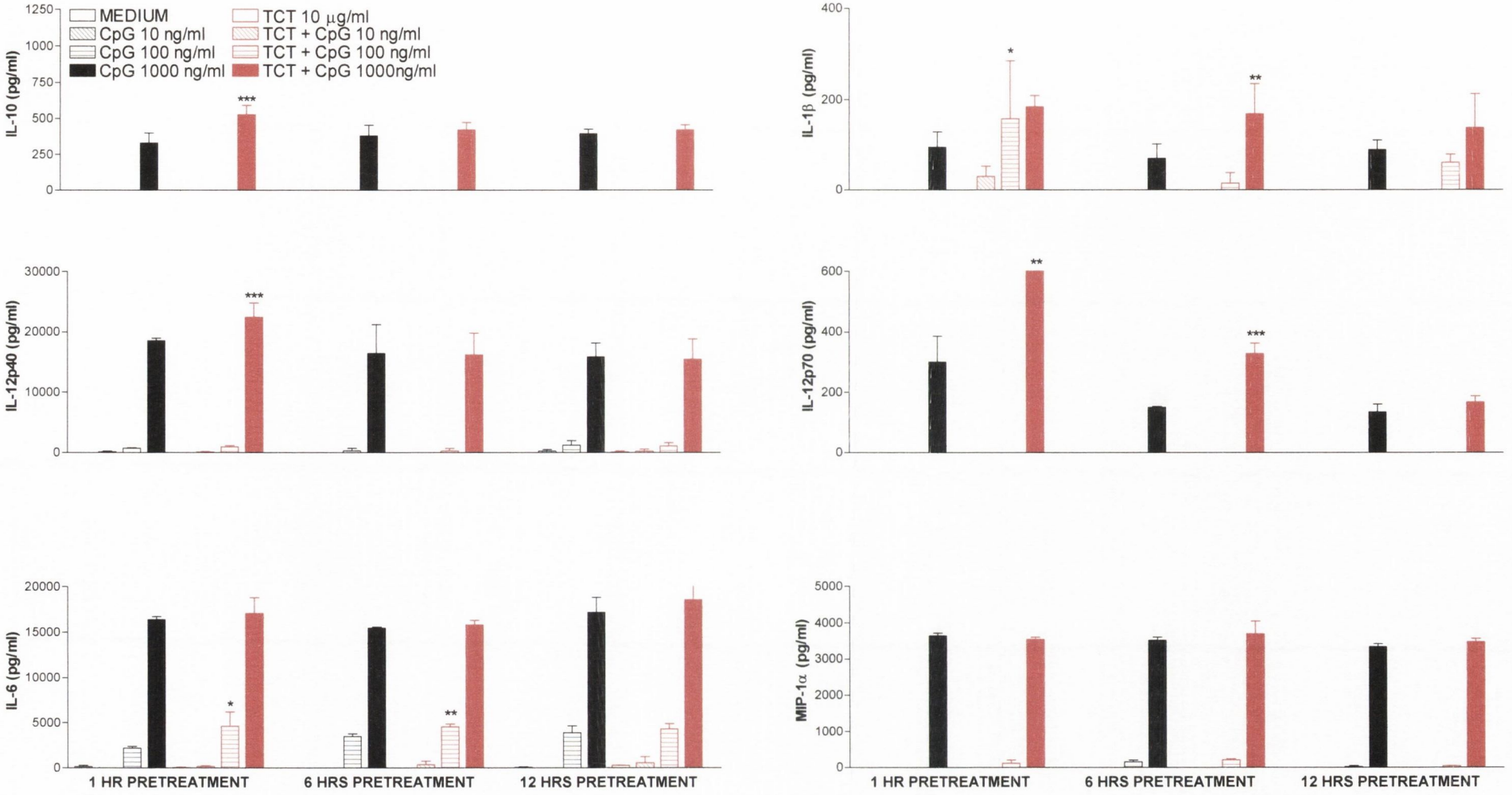

MEDIUM
CpG 10 ng/ml
CpG 100 ng/ml
CpG 1000 ng/ml
TCT 10 μg/ml
TCT + CpG 10 ng/ml
TCT + CpG 100 ng/ml
TCT + CpG 1000ng/ml
IL-10 (pg/ml)
IL-1β (pg/ml)
IL-12p40 (pg/ml)
IL-12p70 (pg/ml)
IL-6 (pg/ml)
MIP-1α (pg/ml)
1 HR PRETREATMENT
6 HRS PRETREATMENT
12 HRS PRETREATMENT

Figure 5.3 Pretreatment of BMDC with TCT enhances IL-1β production in response to TLR-2 Ligand, Pam-3Csk. Bone marrow-derived DC from BALB/c mice were pretreated with TCT (10 μg/ml) for 1, 6 and 12 h before further stimulation with Pam-3Csk (10, 100, 1000 ng/ml) for 12 h. Cytokine and chemokine concentrations were evaluated by ELISA. Values represent means ± SD of triplicate samples. *, $p< 0.05$; **, $p < 0.01$;

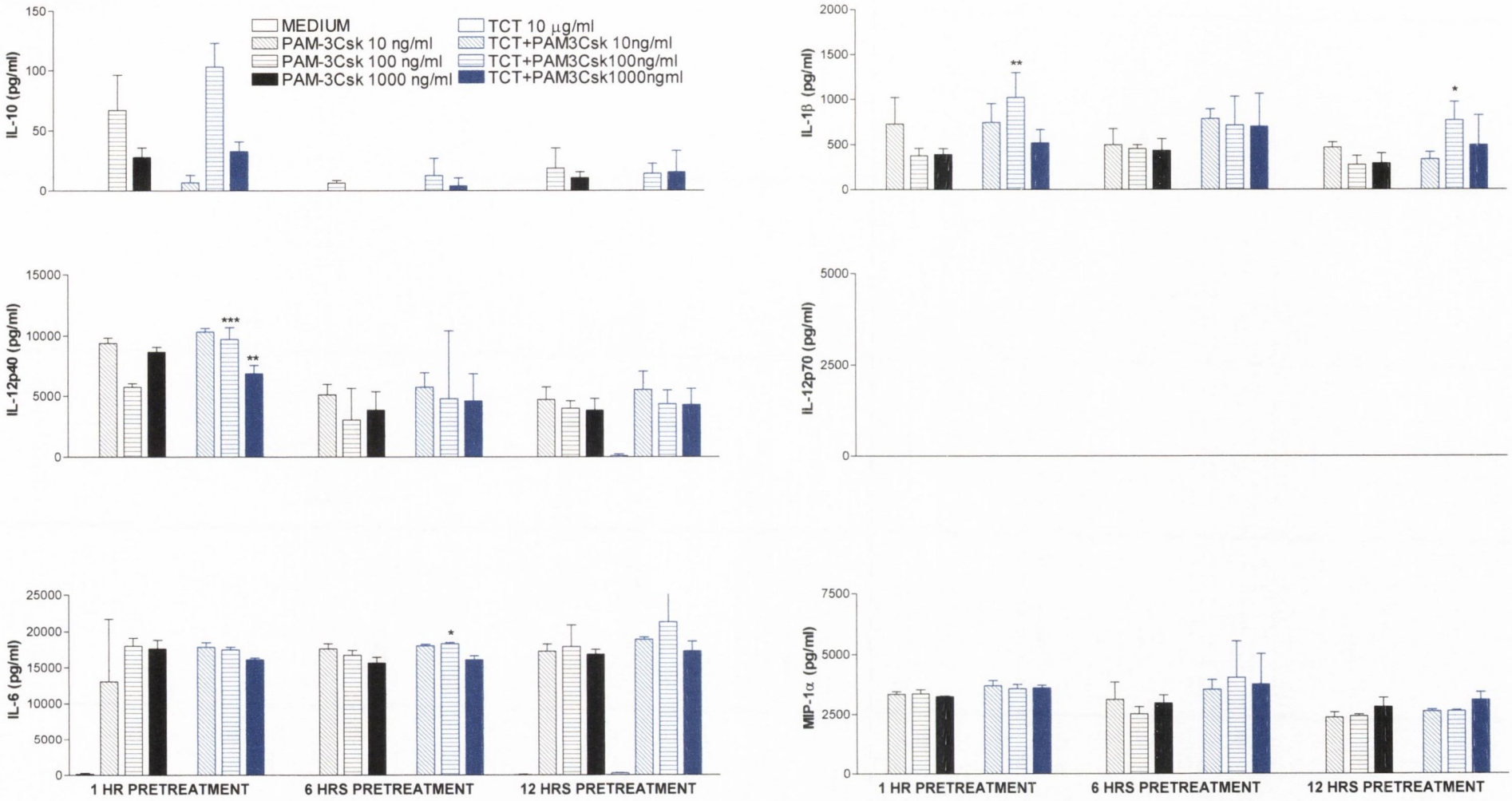
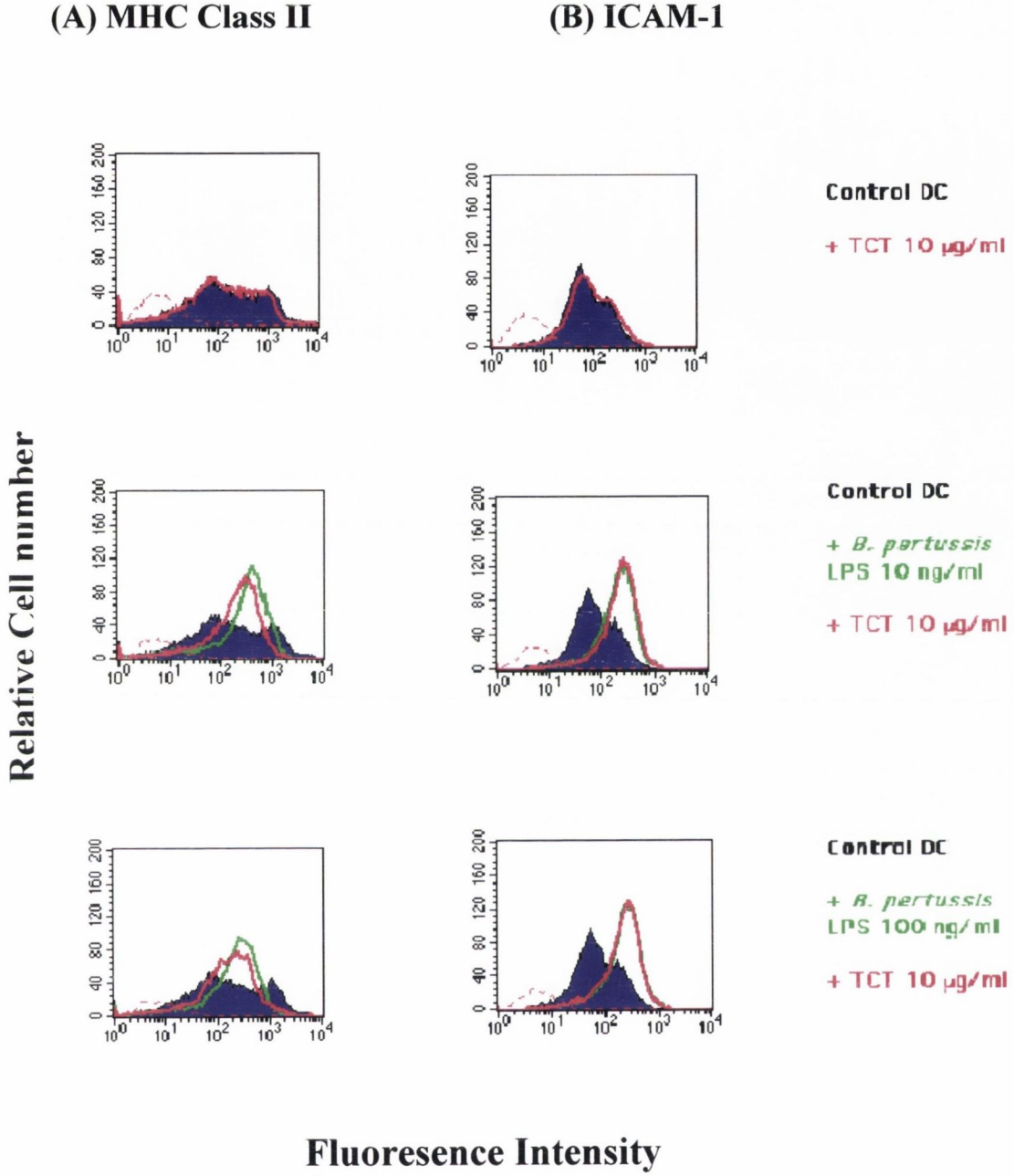
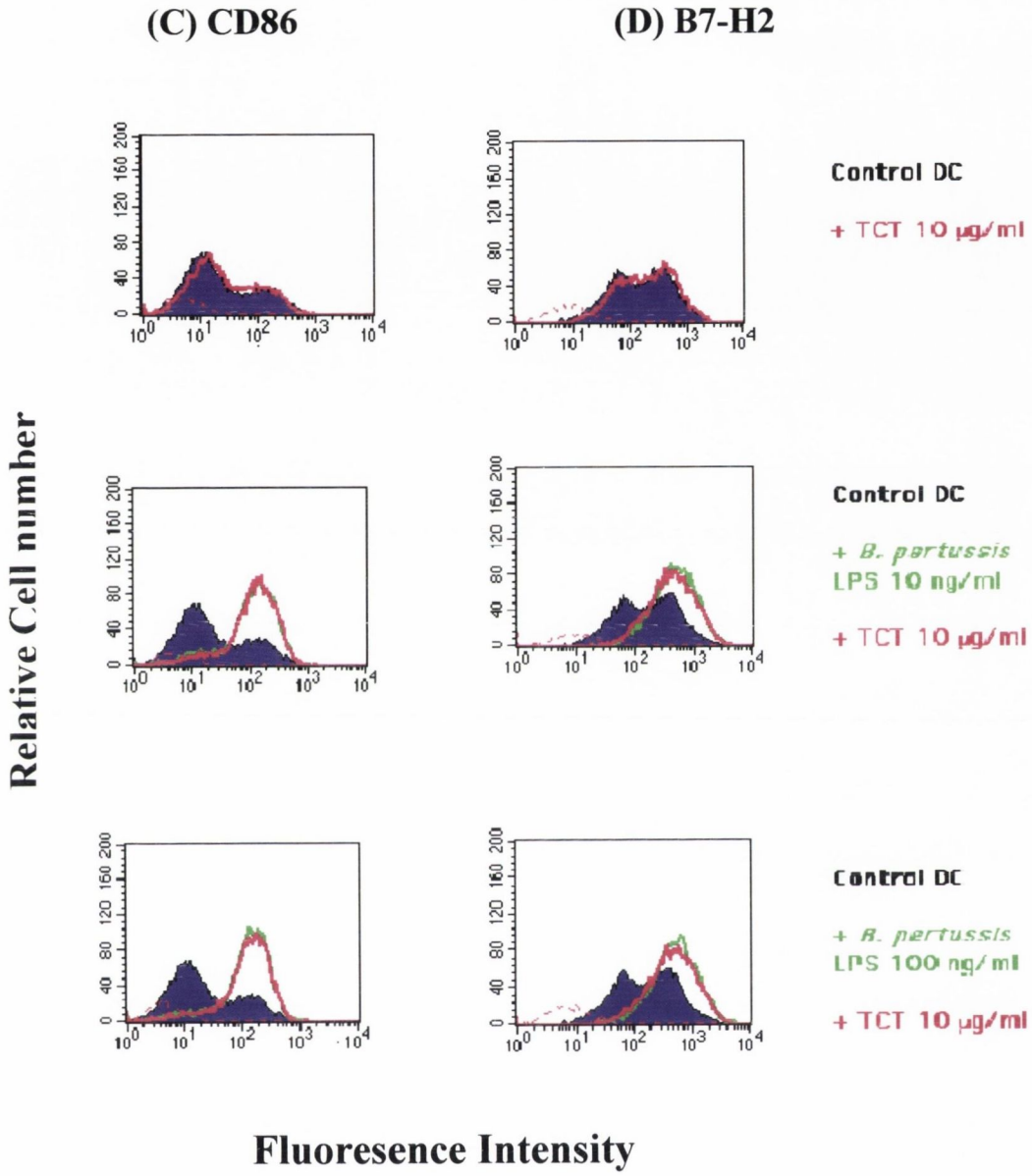

MEDIUM
PAM-3Csk 10 ng/ml
PAM-3Csk 100 ng/ml
PAM-3Csk 1000 ng/ml
TCT 10 μg/ml
TCT+PAM3Csk 10ng/ml
TCT+PAM3Csk100ng/ml
TCT+PAM3Csk1000ngml
IL-10 (pg/ml)
IL-1β (pg/ml)
IL-12p40 (pg/ml)
IL-12p70 (pg/ml)
IL-6 (pg/ml)
MIP-1α (pg/ml)
1 HR PRETREATMENT
6 HRS PRETREATMENT
12 HRS PRETREATMENT

Figure 5.4 TCT downregulates LPS-induced MHC class II expression on DC. Bone marrow-derived DC from BALB/c mice were cultured for 6 h with TCT (10 μg/ml) or medium alone, then stimulated with *B. pertussis* LPS (10 ng/ml, 100 ng/ml) for a further 12 h and then analyzed for surface expression of MHC class II and costimulatory molecule expression by immunofluorescence analysis with Abs specific for MHC Class II (A), ICAM-1(B), CD86 (C) and B7-H2 (C). Results are presented as mean fluorescence intensity on DC treated with **LPS** (green), **TCT plus LPS** (pink) versus **untreated (control) $CD11c^+$ cells** (purple). Relevant isotype control antibodies are plotted as a broken line in the same colour as the treatment.


(A) MHC Class II
(B) ICAM-1
Relative Cell number
Control DC
+ TCT 10 μg/ml
Control DC
+ B. pertussis
LPS 10 ng/ml
+ TCT 10 μg/ml
Control DC
+ B. pertussis
LPS 100 ng/ml
+ TCT 10 μg/ml
Fluoresence Intensity


(C) CD86
(D) B7-H2
Relative Cell number
Control DC
+ TCT 10 μg/ml
Control DC
+ B. pertussis LPS 10 ng/ml
+ TCT 10 μg/ml
Control DC
+ B. pertussis LPS 100 ng/ml
+ TCT 10 μg/ml
Fluoresence Intensity

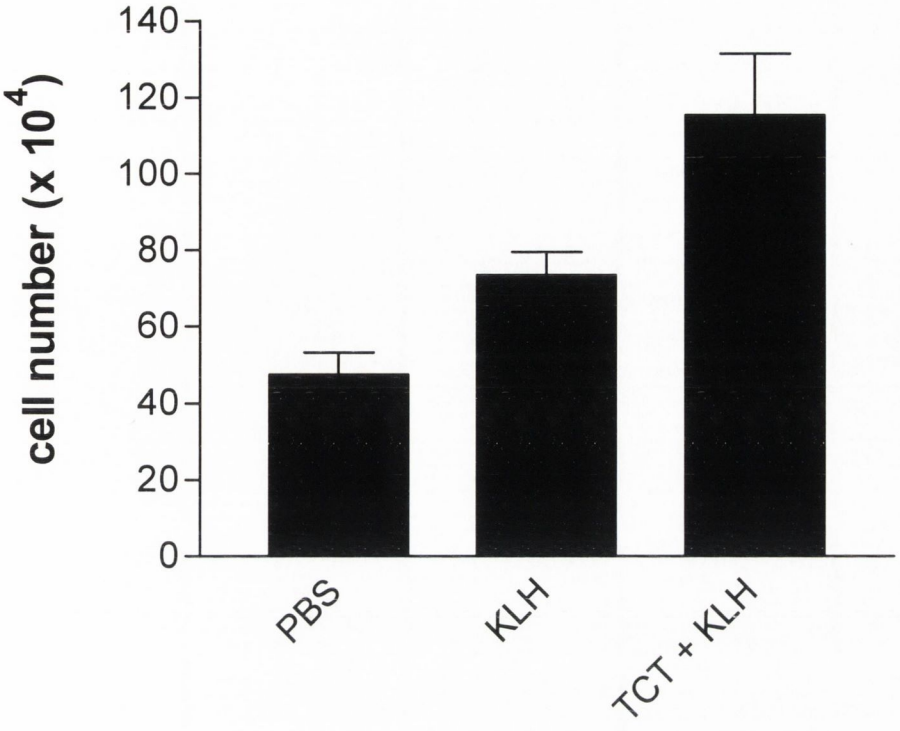
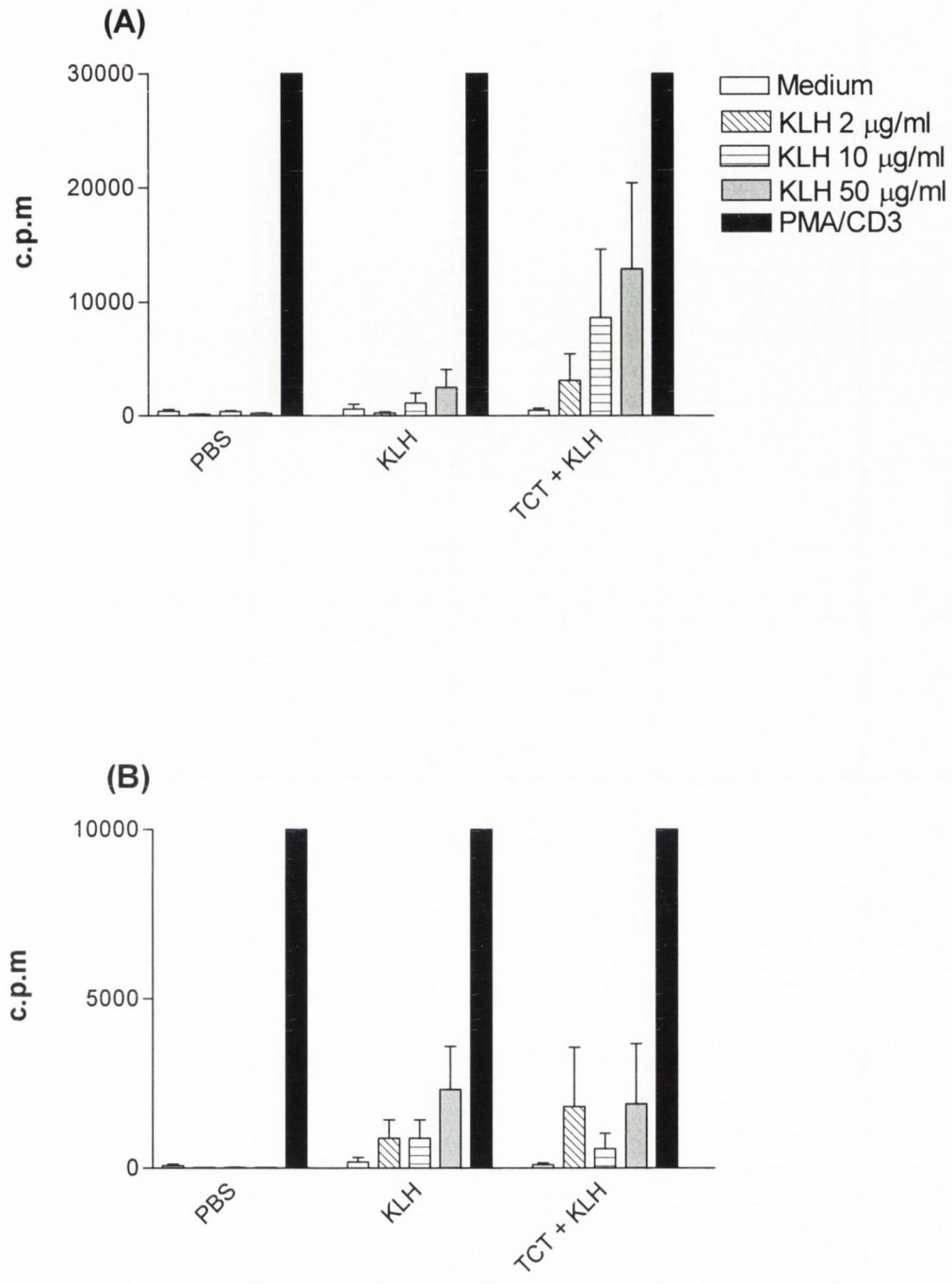


Figure 5.5 TCT enhances the recruitment of cells to the local lymph node following s.c. injection. BALB/c mice were immunized s.c. into 2 sides of the flank with either PBS, KLH (20 μg/mouse) alone or KLH with TCT (10 μg/mouse). Popliteal lymph nodes were isolated 7 d later, homogenized and live cells were counted. Results are expressed as mean (± S.D) cell numbers from 5 mice per group. ** , $P > 0.001$.

Figure 5.6 Antigen-specific T cell proliferation responses enhanced in spleens of mice immunized with TCT. BALB/c mice were injected subcutaneously (into the footpad) with either PBS alone, KLH (20 μg/mouse) or TCT (10 μg/mouse) and KLH (20 μg/mouse). After 7 d, the spleen (A) and popliteal lymph node (B) cells were prepared and stimulated with KLH (2, 10, 50 μg/ml) and PMA/CD3 or medium, were used as positive and negative controls respectively. Supernatants were collected 72h later (for cytokine detection; figures 5.7, 5.8), fresh medium was added to the wells and cells were incubated overnight. At day 4, 2 μCi of ^{3}H thymidine was added to each well. Plates were incubated for a further 6 h, after which cells and ^{3}H incorporation was determined. Results are the mean responses (± SD) for 5 mice assessed individually in triplicate.


(A)
30000
20000
10000
0
c.p.m
Medium
KLH 2 μg/ml
KLH 10 μg/ml
KLH 50 μg/ml
PMA/CD3
PBS
KLH
TCT + KLH
(B)
10000
5000
0
c.p.m
PBS
KLH
TCT + KLH

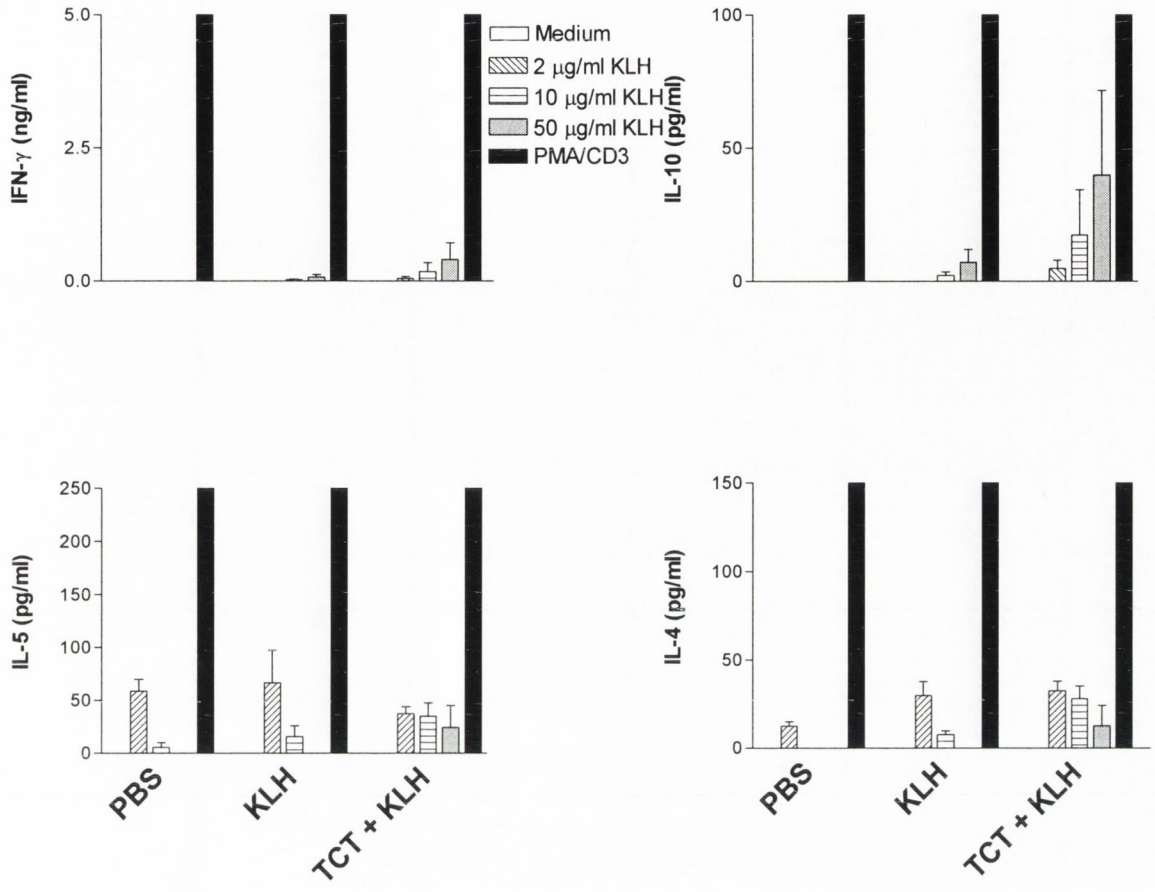


Figure 5.7: TCT fails to enhance KLH-specific T cells responses by spleen cells from BALB/c immunized mice. BALB/c mice were injected subcutaneously into the footpad with either PBS alone, KLH (20 μg/mouse) or TCT (10 μg/mouse) and KLH (20 μg/mouse). 7 d later, the spleens were collected and single cell suspensions cultured in the presence of increasing concentrations of KLH (2, 10, 50 μg/ml), PMA and anti-CD3 or medium only, (positive and negative controls respectively). Supernatants were removed after 3 days and tested for IFN-γ, IL-10, IL-4 and IL-5 by ELISA. Results are mean (± SD) values for 5 mice per group.

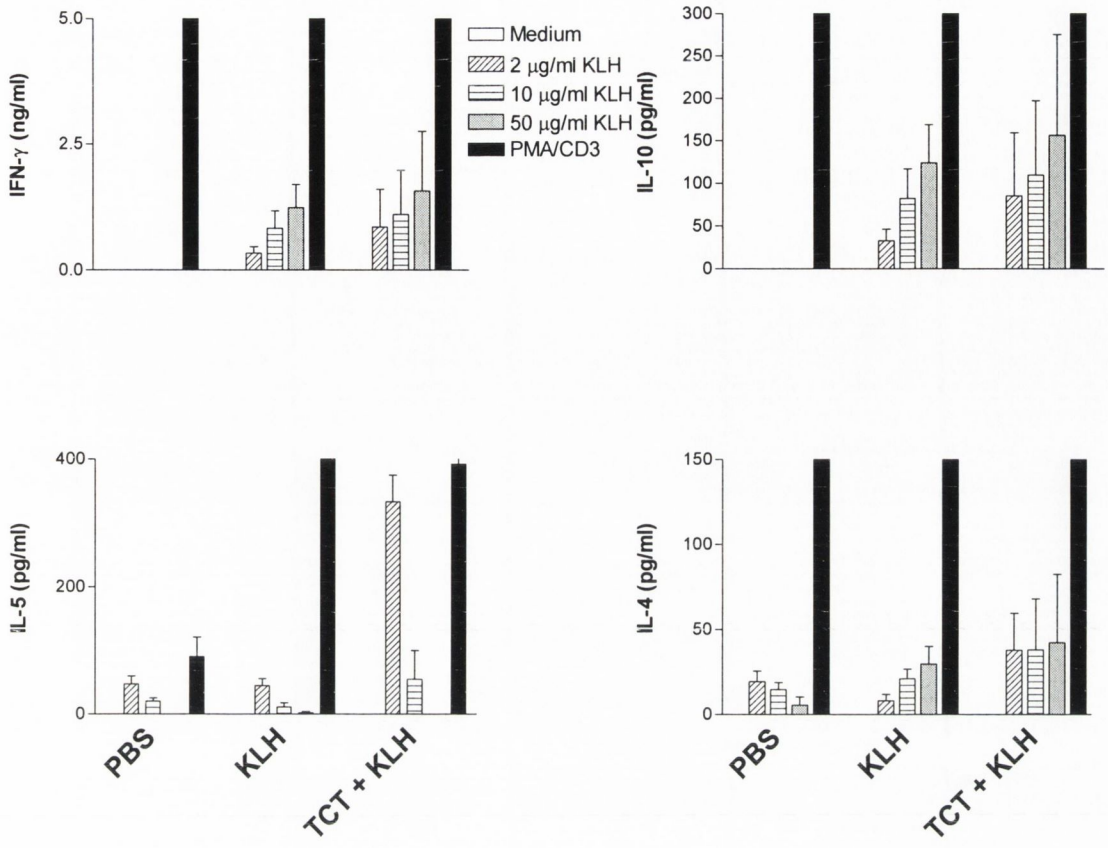
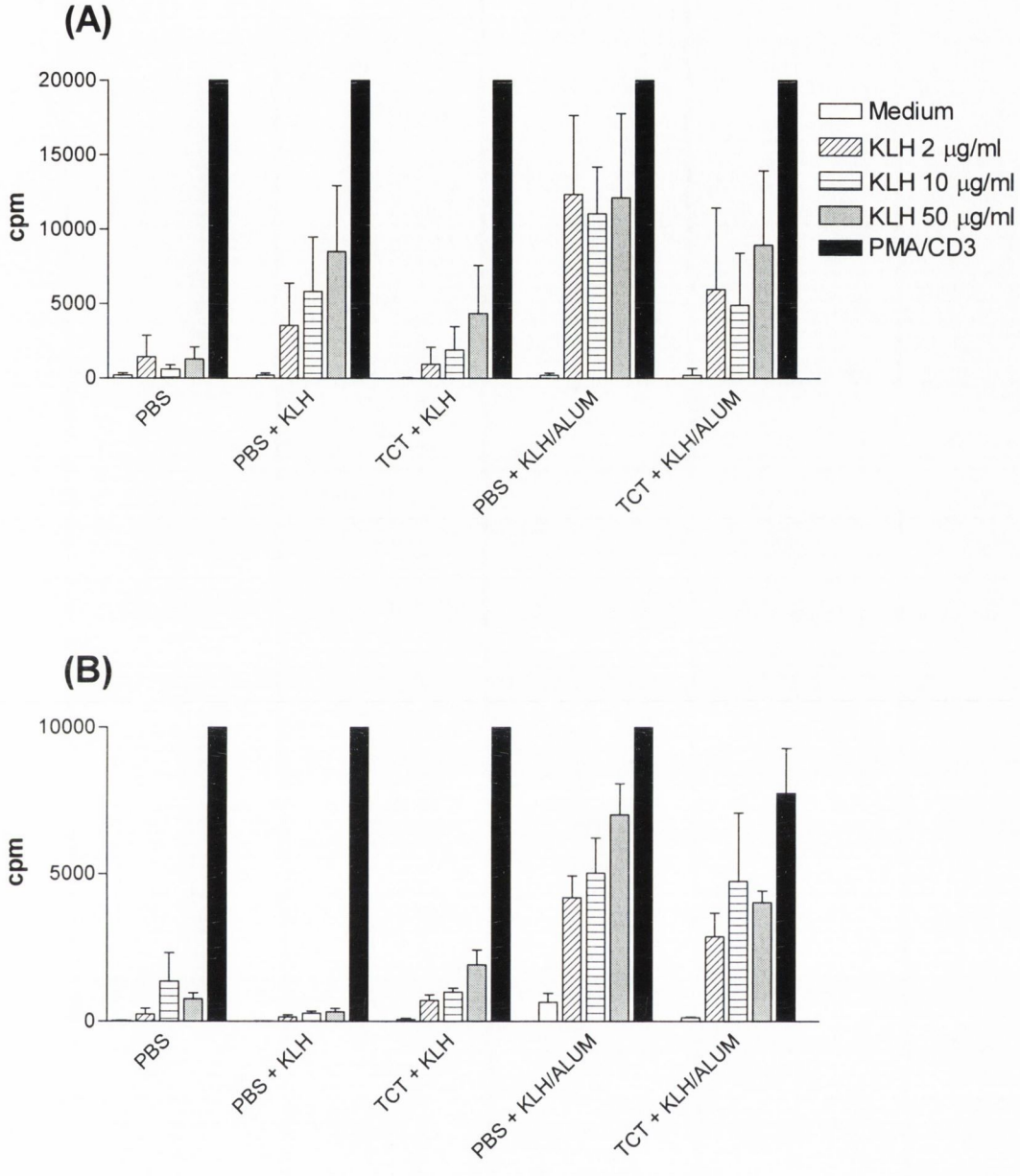


Figure 5.8: TCT has little effect as an adjuvant on cytokine production in lymph nodes of BALB/c immunized mice. Immunisation schedule as described in figure 5.7. 7 d later, the popliteal lymph nodes were collected and single cell suspensions cultured in the presence of graded concentrations of KLH (2, 10, 50 μg/ml) and PMA/CD3 or medium were used as positive and negative controls respectively. Supernatants were removed after 3 days and tested for IFN-γ, IL-10, IL-4, IL-5 by ELISA. Results are mean (± SD) values for 5 mice per group.

Figure 5.9 TCT significantly enhances KLH-specific total IgG antibody titres. BALB/c mice were injected subcutaneously into the footpad with either PBS alone, KLH (20 μg/mouse) or TCT (10 μg/mouse) and KLH (20 μg/mouse). 7 d later, KLH-specific antibody titres were evaluated by ELISA. Results are mean (± SD) values for 5 mice per group. *, $p < 0.01$. KLH versus KLH + TCT

IgG2a (Log10)
IgG1 (Log10)
Total IgG (Log10)
2
3
4
PBS
KLH
TCT+KLH
*

Figure 5.10 Pre-injection with TCT 6 h before immunization with alum adsorbed KLH reduces antigen-specific T cell proliferation responses in the spleen and local lymph nodes. BALB/c mice were injected subcutaneously (into the flank) with either PBS alone, PBS and KLH (20 μg/mouse) or TCT(10 μg/mouse)/KLH (20 μg/mouse). 14 days later, spleenand lymph node cells were collected. Spleen (A) and lymph node (B) cell suspensions were cultured in the presence of various concentrations of KLH (2, 10, 50 μg/ml) and PMA/anti-CD3 or medium were used as positive and negative controls respectively. Supernatants were collected 72h later, fresh medium was added to the wells and cells were incubated overnight. At day 4, 2 μCi of ^{3}H thymidine was added to each well. Plates were incubated for a further 6 h, and ^{3}H incorporation was determined. Results are the mean responses (± SD) for 5 mice assessed individually in triplicate.


(A)
20000
15000
10000
5000
0
cpm
Medium
KLH 2 μg/ml
KLH 10 μg/ml
KLH 50 μg/ml
PMA/CD3
PBS
PBS + KLH
TCT + KLH
PBS + KLH/ALUM
TCT + KLH/ALUM
(B)
10000
5000
0
cpm
PBS
PBS + KLH
TCT + KLH
PBS + KLH/ALUM
TCT + KLH/ALUM

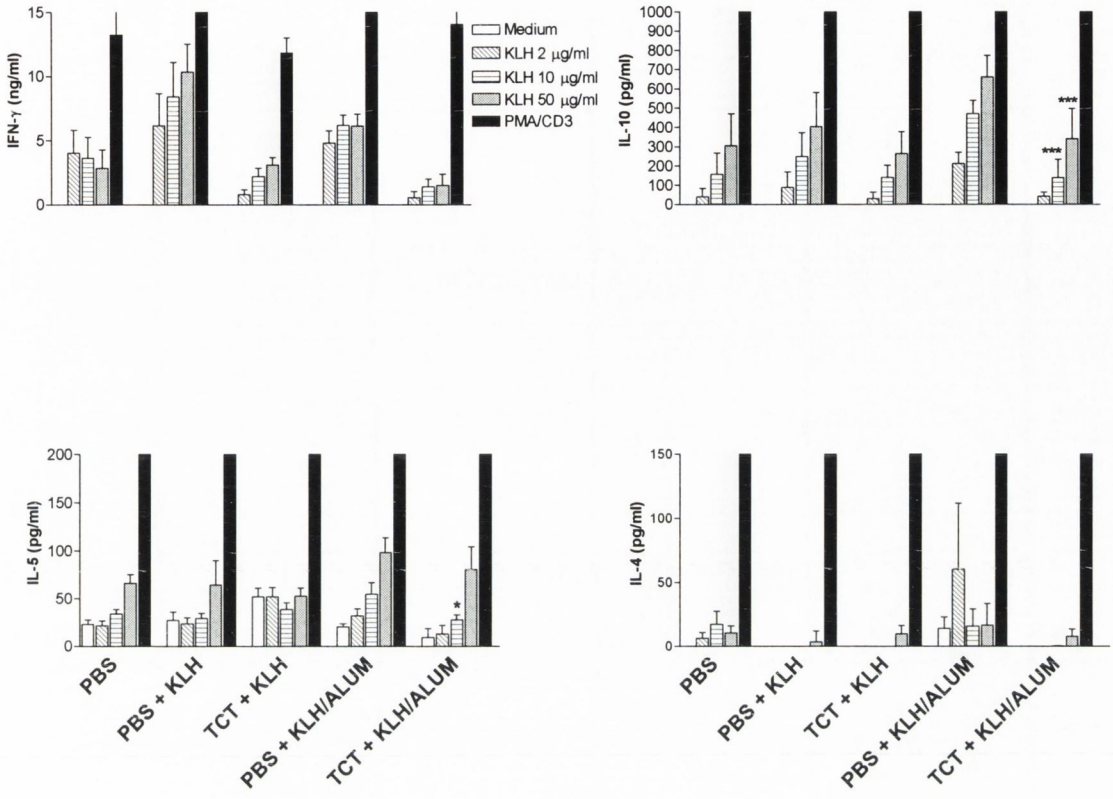


Figure 5.11 TCT impairs splenic antigen-specific T cell responses induced with KLH adsorbed to alum *in vivo*. BALB/c mice were injected subcutaneously into the flank with either PBS alone, PBS and KLH (20 μg/mouse) or TCT (10 μg/mouse) and KLH (20 μg/mouse). 14 days later, the spleens were collected. Single spleen cell suspensions cultured in the presence of increasing concentrations of KLH (2, 10, 50 μg/ml) and PMA/CD3 or medium, (positive and negative controls respectively). Supernatants were removed after 3 days and tested for IFN-γ , IL-10, IL-4 and IL-5 by ELISA. Results are mean (± SD) values for 5 mice per group. *, $p < 0.05$; **, ***, $p < 0.001$.

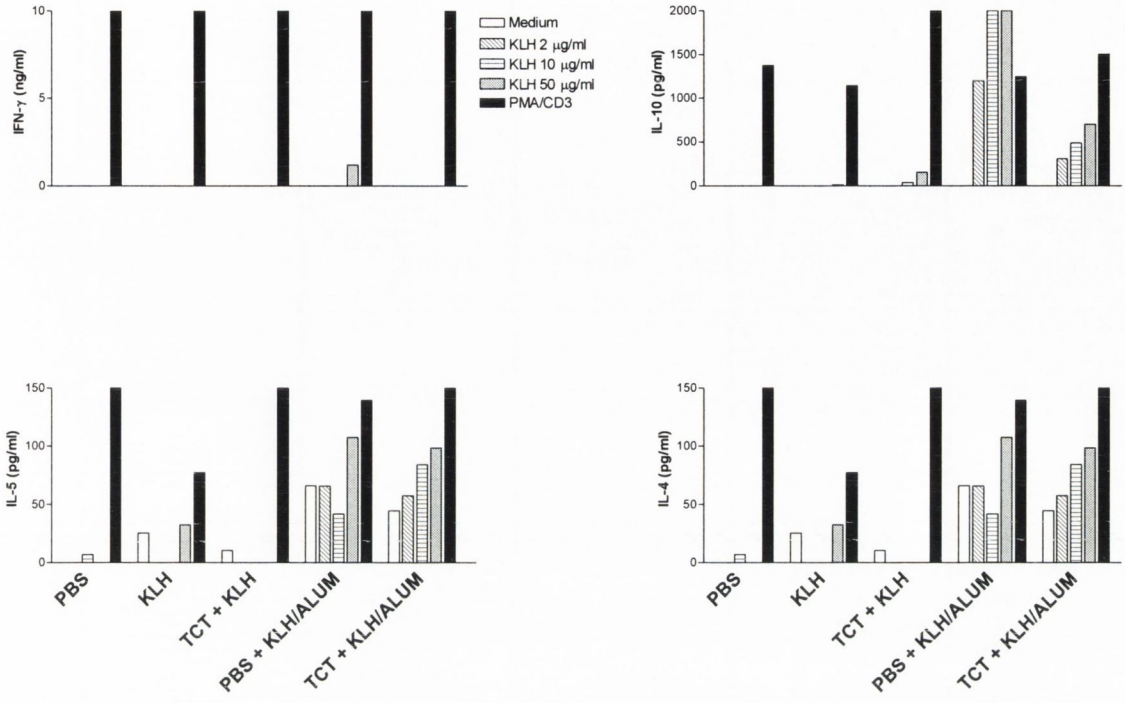


Figure 5.12: TCT impairs antigen-specific T cell responses elicited by Alum in the lymph nodes of immunized mice. BALB/c mice were injected subcutaneously as described in figure 5.11. 14 days later, the inginual lymph nodes were collected. Pooled lymph node cells were cultured in the presence of graded concentrations of KLH (2, 10, 50 μg/ml) and PMA/CD3 or medium, were used as positive and negative controls respectively. Supernatants were removed after 3 days and tested for IFN-γ, IL-10, IL-4 and IL-5 by ELISA. Results are mean (± SD) values for 5 mice per group.

Figure 5.13: TCT does not impair antibody responses to antigen with alum as an adjuvant. BALB/c mice were injected subcutaneously as described in figure 5.10. 14 d after immunisation KLH-specific antibody titres was determined by ELISA. Results are expressed as mean antibody titres (± SD) for 5 mice per group.

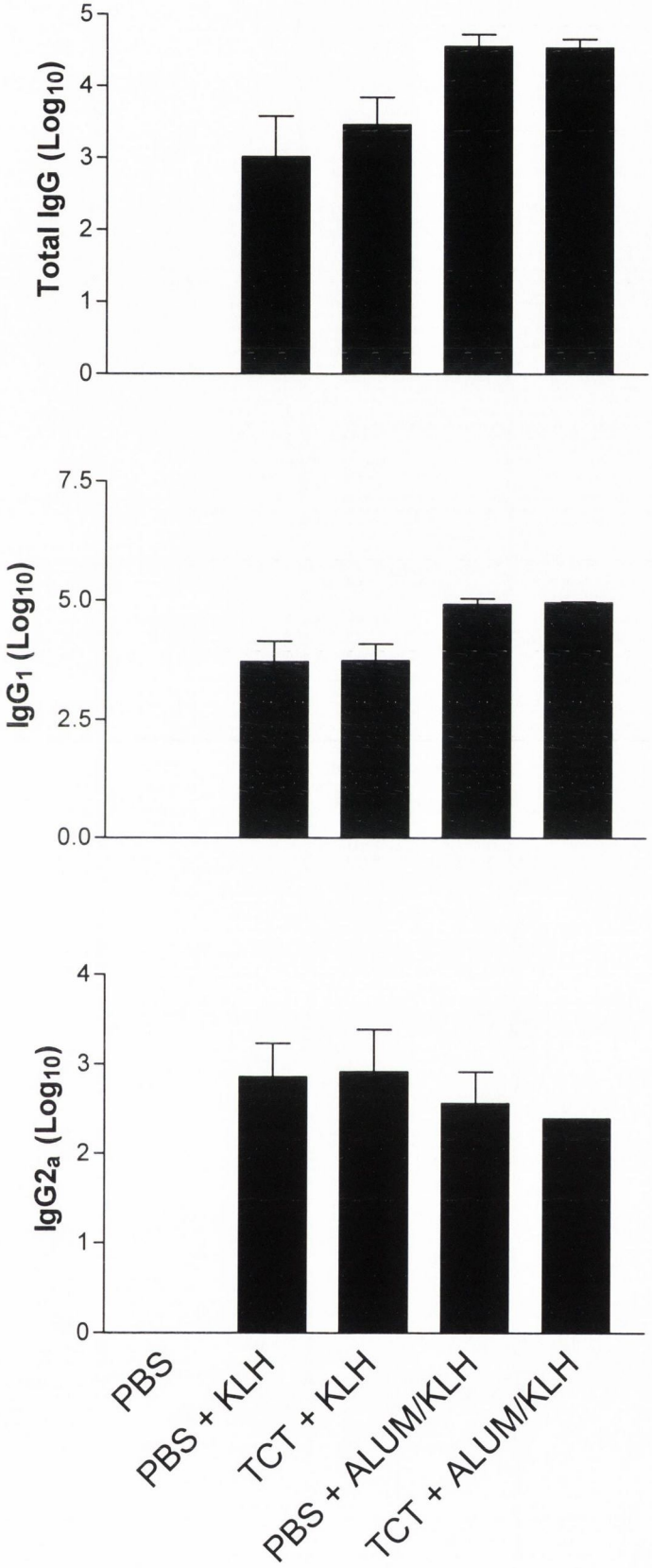
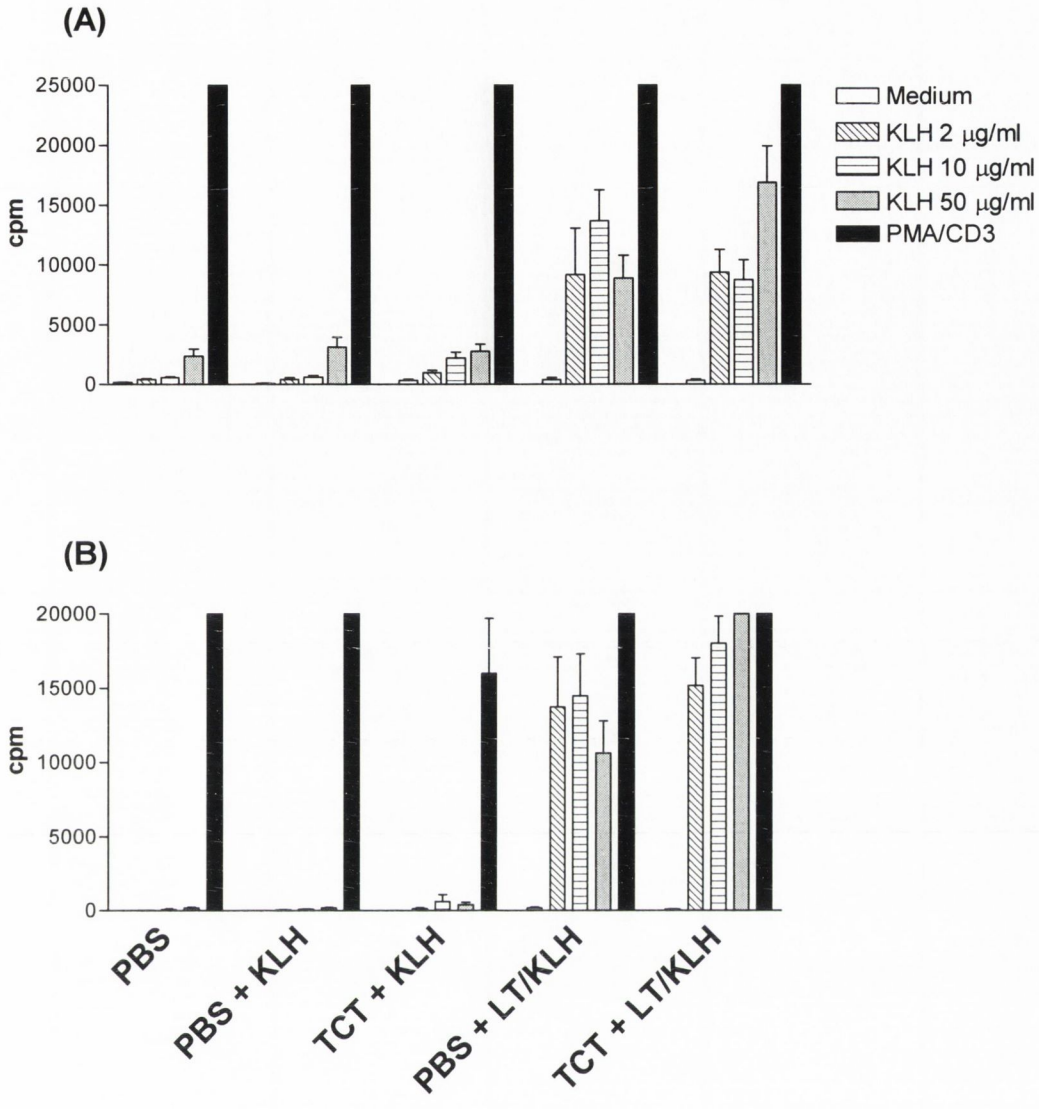

Total IgG (Log10)
5
4
3
2
1
0
IgG1 (Log10)
7.5
5.0
2.5
0.0
IgG2a (Log10)
4
3
2
1
0
PBS
PBS + KLH
TCT + KLH
PBS + ALUM/KLH
TCT + ALUM/KLH

Figure 5.14 TCT treatment does not affect antigen-specific T cell proliferation responses in TCT treated mice immunized with LT. .

BALB/c mice were injected subcutaneously (into the flank) with either PBS alone, PBS and KLH (20 μg/mouse) or TCT(10 μg /mouse)/KLH (20 μg/mouse). 14 days later, the spleens and lymph nodes were collected. Spleen (A) and lymph node (B) cell suspensions were cultured in the presence of various concentrations of KLH (2, 10, 50 μg/ml) and PMA/CD3 or medium were used as positive and negative controls respectively. Supernatants were collected 72 h later, fresh medium was added to the wells and cells were incubated overnight. At day 4, 2 μCi of ^{3}H thymidine was added to each well. Plates were incubated for a further 6 h and ^{3}H incorporation was determined. Results are the mean responses (± SD) for 5 mice assessed individually in triplicate.


(A)
25000
20000
15000
10000
5000
0
cpm
Medium
KLH 2 μg/ml
KLH 10 μg/ml
KLH 50 μg/ml
PMA/CD3
(B)
20000
15000
10000
5000
0
cpm
PBS
PBS + KLH
TCT + KLH
PBS + LT/KLH
TCT + LT/KLH

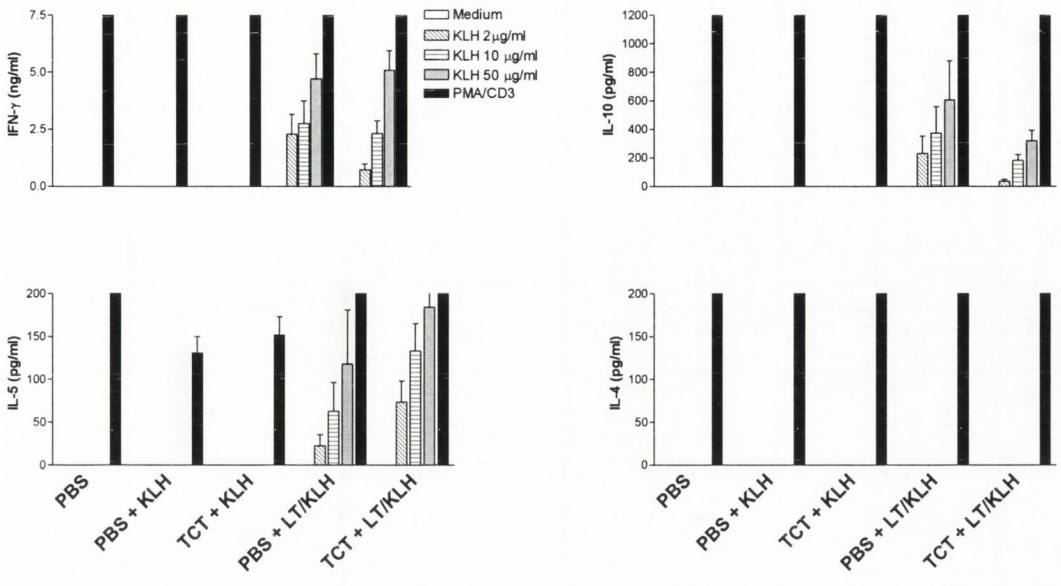


Figure 5.15: TCT inhibits antigen-specific IL-10 production by spleen cells in mice immunised with antigen with LT as an adjuvant. BALB/c mice were pre-treated by subcutaneous (s.c) injection with either PBS or TCT (10 µg/mouse) and then immunised s.c. with KLH (20 µg/mouse) alone or with LT (10 ng/mouse) 6 h later. 14 days later, spleens were collected and spleen cell suspensions (2 x 10^6 cells/ml) cultured in the presence of KLH (2, 10 and 50 µg/ml). Supernatants were removed after 3 days and concentrations of IFN-γ, IL-10, IL-4 and IL-5 were evaluated by ELISA. Results are mean (±SD) values for 5 mice per group.

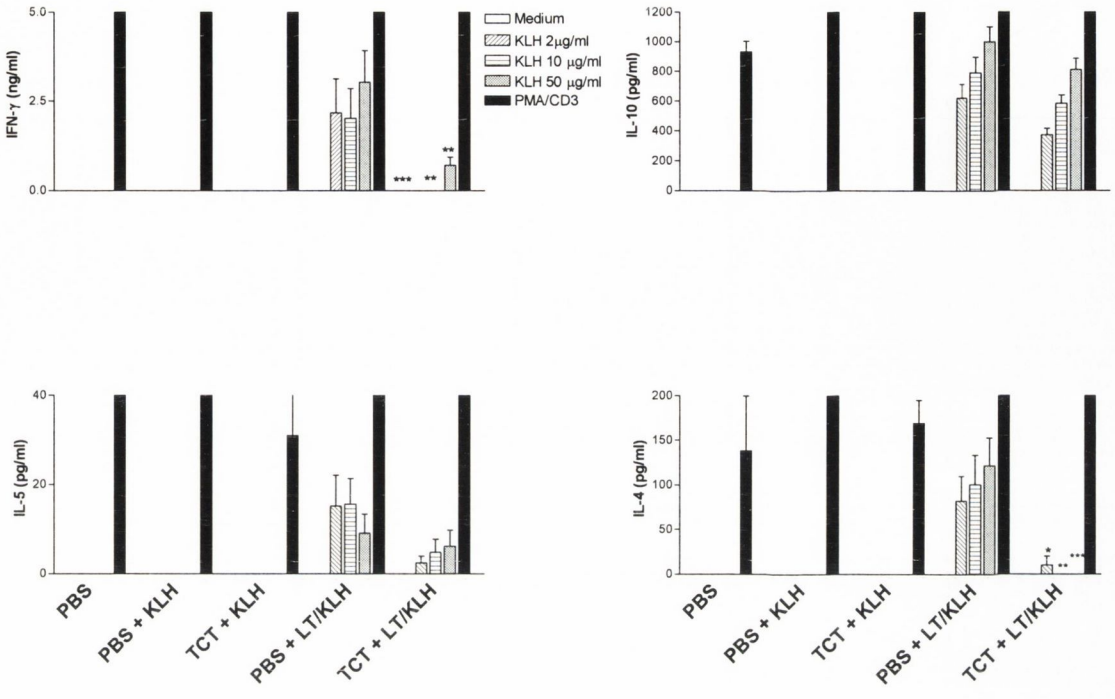
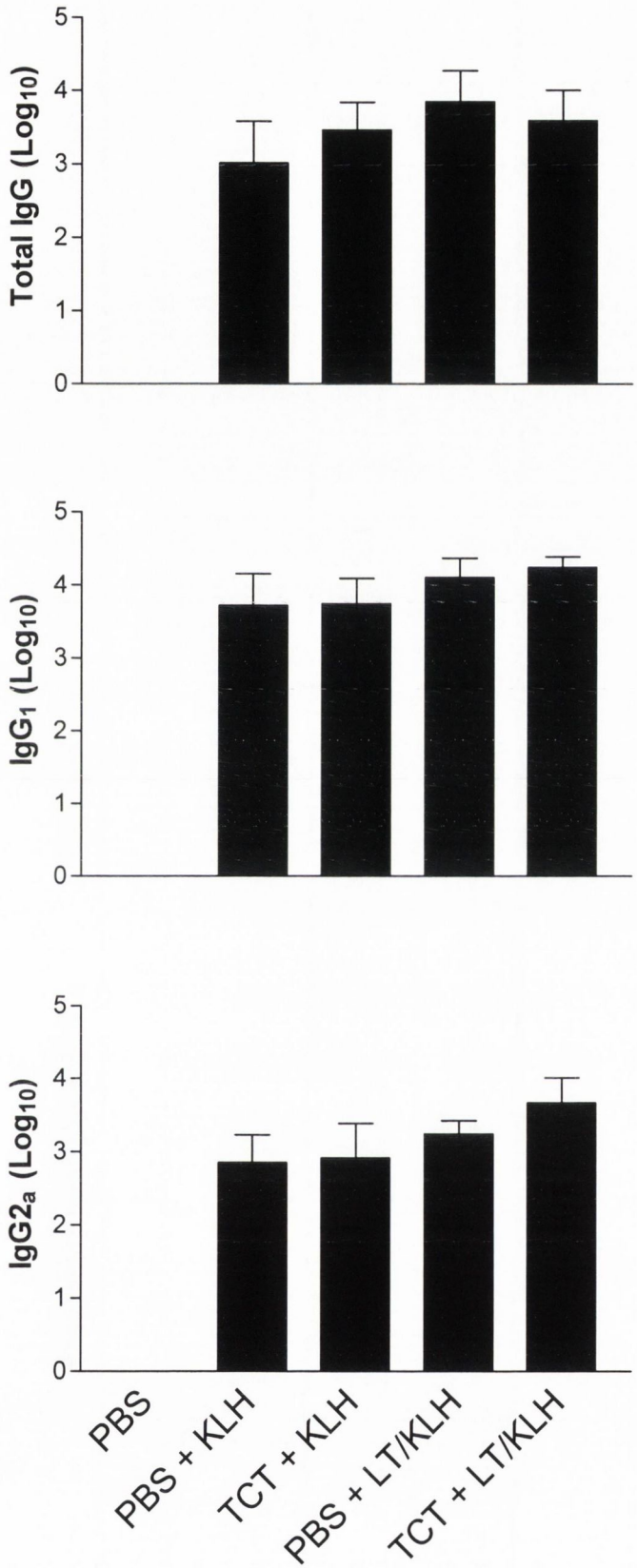


Figure 5.16: TCT impairs antigen-specific T cell responses in lymph node cells of mice immunised with antigen and LT as an adjuvant. BALB/c mice were injected subcutaneously as described in figure 5.15. 14 days later, the inguinal lymph nodes were collected and lymph node cell suspensions (1 x 10^6 cells/ml) cultured in the presence of KLH (2, 10 and 50 μg/ml). Supernatants were removed after 3 days and concentrations of IFN-γ, IL-10, IL-4 and IL-5 were evaluated by specific ELISA. Results are mean (±SD) values for 5 mice per group. *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$ 6 h PBS s.c. v 6 h TCT s.c.

Figure 5.17: TCT does not impair antibody responses to antigen with LT as adjuvant. BALB/c mice were injected subcutaneously as described in figure 5.15. 14 d after immunization KLH-specific antibody titres was determined by ELISA. Results are expressed as mean antibody titres (± SD) for 5 mice per group.


Total IgG (Log10)
IgG1 (Log10)
IgG2a (Log10)
0
1
2
3
4
5
PBS
PBS + KLH
TCT + KLH
PBS + LT/KLH
TCT + LT/KLH

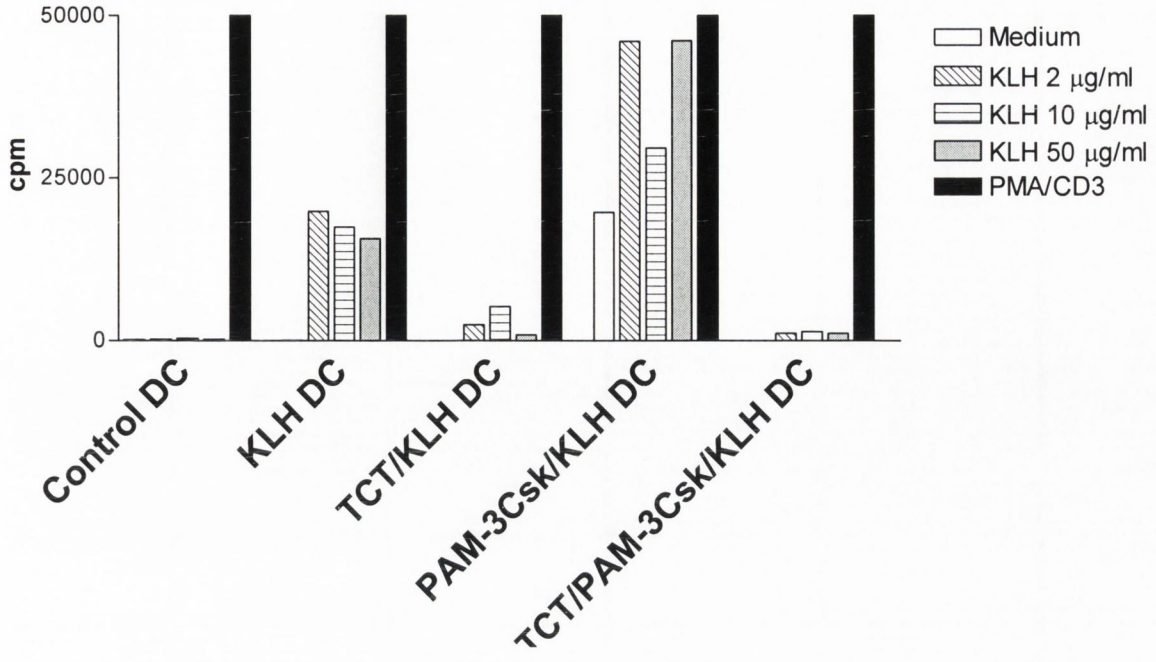


Figure 5.18: Adoptive transfer of TCT pretreated DC impairs KLH-specific T cell proliferation in the lymph nodes. DC were stimulated with KLH (20 μg/ml), or KLH with TCT (10 μg/ml), Pam-3CSK (500 ng/ml) or TCT plus Pam-3CSK. Cells were treated for 2 h, washed 5 times (to remove all traces of each stimulus) and 1 x 10^5cells were injected s.c into the flank of naïve mice. Cellular responses in local inguinal lymph nodes were assessed 14 d after cell transfer. Lymph node cells from each treatment group were pooled and cultured in the presence of KLH (2, 10 and 50 μg/ml) or PMA/CD3 or medium, as positive and negative controls respectively. Supernatants were collected 72h later for cytokine analysis, see figure 5.19, fresh medium was added to the wells and cells were incubated overnight. At day 4, 2 μCi of ^{3}H thymidine was added to each well. Plates were incubated for a further 6 h, after which cells and ^{3}H incorporation was determined. Results are the mean responses (± SD) for pooled lymph node cells of 5 mice assessed in triplicate.

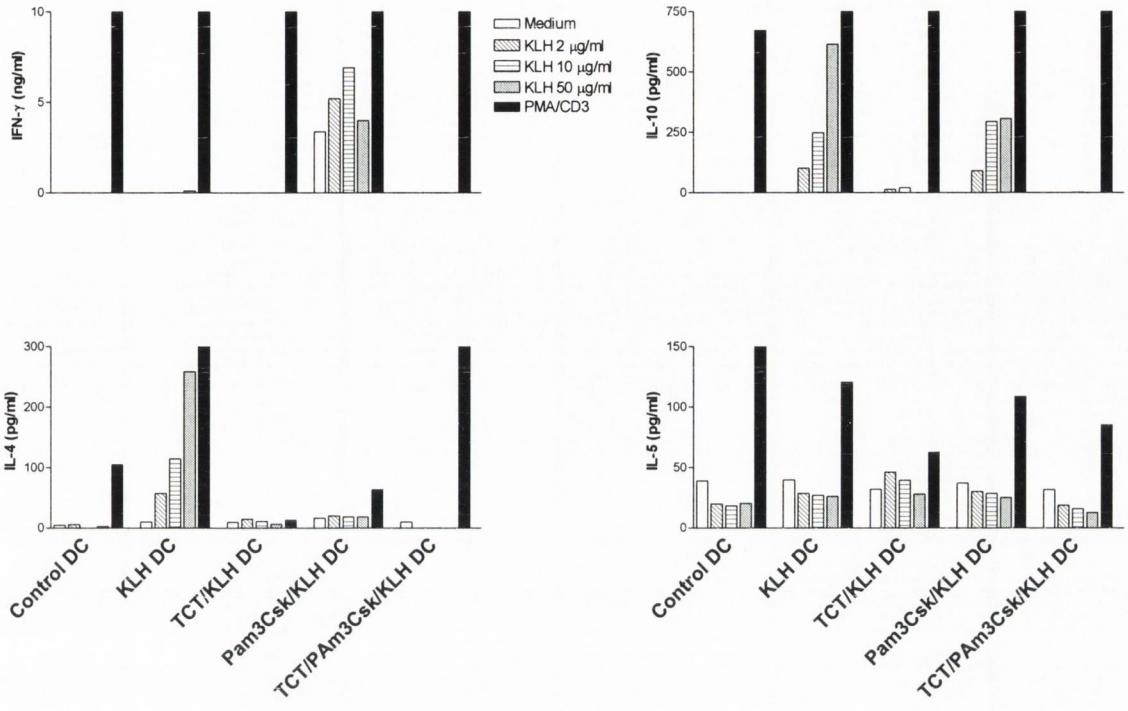


Figure 5.19: Adoptive transfer of TCT pretreated DC modulates the antigen-specific cytokine response to Pam-3Csk *in vivo*. DC were stimulated with KLH (20 µg/ml), or KLH with TCT (10 µg/ml), Pam-3Csk (500 ng/ml) or TCT plus Pam-3Csk. Cells were treated for 2 h, washed 5 times (to remove all traces of each stimulus) and injected s.c into the flank into naïve mice (1 x 10^5/mouse). Cellular responses in local inguinal lymph nodes were assessed 14 d after cel transfer. Lymph node cells from each treatment group were pooled and cultured in the presence of KLH (2, 10 and 50 µg/ml) or PMA/CD3 or medium only, as positive and negative controls respectively. Supernatants were removed after 72 h and concentrations of IFN-γ, IL-10, IL-4 and IL-5 were evaluated by specific ELISA. Results are for pooled lymph node cells from 5 mice per group assessed in triplicate.

Chapter 6

General Discussion

6.0 GENERAL DISCUSSION

During the past decade, a dramatic shift has occurred in our understanding and appreciation of innate immunity. The identification of TLRs as essential regulators of mammalian host defence has fundamentally altered our perception of innate immunity. The innate immune system is no longer regarded to be of secondary importance in the hierarchy of immune functions. It is now recognised as a discriminating system that forms the first line of defence against an invading pathogen and has a decisive impact on the outcome of the subsequent immune response.

Until recently, studies examining the immune response to *B. pertussis* focused on the adaptive immune response to the pathogen. This was largely due to the fact that *B. pertussis* was considered as an exclusively extracellular pathogen and that protection against *B. pertussis* was mainly mediated by the humoral arm of the immune system, including the production of antibodies. A role for both B and T cell components of the adaptive immune response was evident in the murine respiratory model of *B. pertussis* infection. Athymic mice failed to clear a *B. pertussis* infection from their lungs, whereas normal BALB/c mice resolved the infection after 35 days (Mills *et al.*, 1993). Immunoglobulin deficient mice also failed to clear a *B. pertussis* infection suggesting a role for antibodies in mediating bacterial clearance (Mahon *et al.*, 1997). The recognition of an intracellular niche in macrophages during *B. pertussis* infection by Cheers and Gray (1969) was the first clue that innate immunity had a role to play in the hosts defence strategy against *B. pertussis* infection (Cheers and Gray, 1969). Subsequently, *B. pertussis* has been shown to invade and survive many mammalian innate cell types including macrophages, PMNs, and respiratory epithelial cells (Bassinet *et al.*, 2000; Friedman *et al.*, 1992; Hellwig *et al.*, 1999;

Saukkonen *et al.*, 1991; Steed *et al.*, 1991; Weingart and Weiss, 2000). The virulence factor, CyaA has been shown to facilitate *B. pertussis* survival within macrophages by inhibiting certain killing mechanisms (Masure, 1993; Mouallem *et al.*, 1990; Steed *et al.*, 1992). *B. pertussis* is however, rapidly killed in human neutrophils and IFN-γ has been shown to enhance the ability of macrophages to kill *B. pertussis in vitro* (Lenz *et al.*, 2000; Mahon *et al.*, 1997).

A critical role for TLRs in host defence against gram-negative bacteria has been strongly suggested by studies undertaken in mice defective in TLR function. TLR4 was identified as the major component of the LPS signalling complex on innate cells (Poltorak *et al.*, 1998a). However, LPS from certain gram-negative bacteria, such as *Leptospira interrogans* and *Helicobacter pyroli* have been reported to signal through TLR2 (Smith *et al.*, 2003; Werts *et al.*, 2001). This study has reported that LPS from *B. pertussis* stimulates pro-inflammatory cytokine and chemokine production from DC and induce maturation of DC through TLR4, suggesting that TLR4 is of major importance in the innate defence against this bacterium. However, consistent with the finding that TLR4 is not required for full maturation of DC by *Salmonella typhimurium*, other components of *B. pertussis* bacterium were capable of inducing DC maturation independently of TLR4 (Rescigno *et al.*, 2002).

The defective innate response to *B. pertussis* in cells from TLR4-defective mice *in vivo* was reflected in an impaired inflammatory cytokine response in the lungs of C3H/HeJ mice soon after *B. pertussis* infection. The transient peak of IL-1β, TNF-α, MIP-1α, and MIP-2 observed in the lungs of the C3H/HeN mice 3 h after *B. pertussis* challenge was not detected in the C3H/HeJ mice, demonstrating that the initial inflammatory response is much reduced in the TLR4-defective mice, indicating an attenuated innate immune response. Consistent with the defective innate

inflammatory response after infection, an increased bacterial burden was observed in the TLR4-defective mice. A compromised innate immune response in the C3H/HeJ mice led to exacerbated *B. pertussis* infection. These findings are consistent with those reported from a recent study, which showed that infection with *Haemophilus influenzae* in TLR-deficient mice exhibited reduced bacterial clearance from the lungs. This was associated with an impaired innate immune response characterised by neutrophils infiltration into the lung and impaired pro-inflammatory cytokine production (Wang *et al.*, 2002). Similar findings have been demonstrated in other models of respiratory infections, including *M. tuberculosis*, and respiratory syncytial virus (Abel *et al.*, 2002; Kurt-Jones *et al.*, 2000).

The consequences of a defective innate immune response not only has effects on bacterial clearance but also has implications for the prevention of local inflammation and immunopathology. The peak of infection in TLR4-defective mice was marked by enhanced cellular infiltrate, local pathology and enhanced IL-1β, MIP-1α, and MIP-2 production in the lungs. In addition, enhanced serum antibody concentrations and *B. pertussis* specific IFN-γ production by spleen cells were higher at the acute stage of infection in C3H/HeJ mice compared with C3H/HeN mice. Enhanced adaptive responses in the TLR4-defective mice along with decreased antigen-specific IL-10 production may reflect a reduction in the number and function of Tr cells. Indirect evidence in support of a protective role for IL-10 producing Tr in host defence against bacteria-induced immune pathology has come from other studies showing increased disease severity in IL-10 deficient mice. IL-10 deficient mice are more susceptible than wild-type mice to infection with *Listeria monocytogenes*; the IL-10 deficient mice have increased inflammatory cellular infiltrate, increased production of pro-inflammatory cytokines in the brain and increased severity of brain

lesions (Deckert *et al.*, 2001). IL-10 deficient mice infected with *H. hepaticas* develop severe inflammation associated with an exacerbated Th1 response (Kullberg *et al.*, 2001).

While antigen-specific IL-10 may be produced by Tr cells, induced during infection to limit inflammation and collateral damage to host tissue, IL-10 can also be produced by innate immune cells in response to pathogen-derived products. Recently, it was reported that FHA and CyaA from *B. pertussis* stimulates IL-10 production by DC and macrophages, and thereby promote the development of Tr1 cells (McGuirk *et al.*, 2002; Mills and McGuirk, 2004; Ross *et al.*, 2004). FHA was also found to enhance LPS-activated IL-10 from DC and macrophages (McGuirk and Mills, unpublished observations). Thus, it is reasonable to propose that in normal C3H/HeN mice, LPS signalling through TLR4 in co-operation with FHA or CyaA or other bacterial molecules enhances the production of IL-10, which transiently inhibits IL-12 and Th1 responses and promotes the development of Tr1 cells. However, TLR4-defective mice have reduced IL-10 production by DC and macrophages and are slower to generate Tr1 cells during a *B. pertussis* infection when compared with the C3H/HeN mice.

Compromised innate immunity therefore, has detrimental effects on the severity of *B. pertussis* infection. Recently, a study by Mann and colleagues (2004) reported the importance of TLR4 in the pro-inflammatory response to another member of the Bordetellae family, *Bordetella bronchiseptica* (Mann *et al.*, 2004). Enhanced immunopathology was observed in the TLR4-defective mice infected with *B. bronchiseptica*. Therefore, the absence of TLR-mediated signalling and as a consequence a defective innate immune response therefore, has a significant outcome on the overall protective immune response to an invading pathogen. This suggests

that, depending on the severity of the infection, the host immune system can produce an appropriate regulatory response to limit immune pathology by transiently inhibiting inflammatory and Th1 responses. In the absence of TLR4 signalling, which appears to be a major mediator not only for innate inflammatory cytokines but also for innate IL-10, the host is unable to mount effective regulatory responses, which can control infection-induced immunopathology.

A compromised innate immune response was also shown to have a profound effect on vaccine-induced immunity to *B. pertussis*. While the precise mechanism of protective immunity to *B. pertussis* induced by immunisation with Pw or Pa remain to be fully defined, it appears that Pa protect largely through antibody induction whereas Pw, which are more effective, induces both antibody and cell mediated immunity. However, there have been few attempts to examine the role of the innate immune system in pertussis vaccine-induced immunity. Furthermore, the role of TLRs in pertussis vaccine-induced immunity has not been addressed. TLR4-defective mice immunised with Pw failed to resolve a *B. pertussis* infection within 14 days of challenge whereas C3H/HeN mice completely cleared the infection from their lungs within 10 days of challenge. This demonstrated an absolute requirement for TLR4 in mediating Pw-induced protection against *B. pertussis*. Although protection was observed in the TLR4-defective mice immunised with Pa, this protection was sub-optimal when compared with that seen in C3H/HeN mice. Similar to the course of *B. pertussis* infection in un-immunised mice, antigen-specific IL-10 production was defective in spleen cells from TLR4-defective mice immunised with both Pa and Pw. This suggests that impaired TLR4-mediated signalling compromises the ability of the innate immune system to produce IL-10 and thereby results in defective adaptive IL-10 production, which was shown to be important in the control of lung inflammation

caused by *B. pertussis* infection. This provided further evidence for the crucial link between innate and adaptive immune response in protection against *B. pertussis*. Vaccines are designed to generate robust immunity through the activation of innate as well as the adaptive arm of the immune response, with the DC playing a pivotal link in this process. In this study, DC from the TLR4-defective mice had defective IL-1β, TNF-α, and IL-12p40 responses to Pw and Pa *in vitro*. This suggests that innate immune cells are activated through TLR4 following immunisation with Pw and Pa. Despite the fact that Pw is comprised of approximately 3000 *B. pertussis* proteins (Geier and Geier, 2004), the response to Pw was severely compromised in DC from TLR4-defective mice. The Pa also induced pro-inflammatory cytokine production from DC and this too was compromised in DC from TLR4-defective mice but not to the same extent as that observed with Pw, suggesting an important role for the contaminating LPS or other TLR4 binding ligands.

Despite the relatively high efficacy of Pw in protecting against severe pertussis in infants, Pw has been associated with a number of mild to severe side effects for many decades. This study and a previous report (Bannatyne and Jackowski, 1987) has addressed the LPS content of pertussis vaccine, and has demonstrated that LPS may play a central role in the protective efficacy of Pw. LPS has long been known to have potent immunomodulatory and adjuvant activity but its direct clinical use is limited due to its extreme toxicity. The generation of protective immunity by vaccination is dependent on the ability of adjuvants, either endogenous as components of killed/attenuated bacteria or viruses, or exogenous (e.g. alum) to boost the adaptive immune responses to protein antigens. Recent studies have suggested that a major function of an adjuvant is to boost innate immunity through activation of DC. However, many pathogen components that have adjuvant activity

are potentially toxic. Recently, Jackson and co-workers synthesised structures containing Th epitopes coupled to B cell target epitopes, linked via a lipid moiety (Pam2Cys) which served to activate DC through the binding and activation of TLR2 (Jackson *et al.*, 2004). Providing the appropriate signals for DC maturation and the inflammatory cytokine release is now recognised as a critical property of the most potent adjuvants. The addition of lipid A derivatives with reduced toxicity but with potent immunomodulatoy properties, such as monophosphoryl lipid A (MLA), a derivative of lipid A from *Salmonella minnesota* RC595 (which mediates its effects through TLR4), could substantially improve the efficacy of some sub-unit vaccines such as Pa, while still retaining the safety profile of these vaccines (Persing *et al.*, 2002). As the mechanisms involved in initiation of immune responses and the role played by the DC and TLR expression are further elucidated, the design of rational safe and effective vaccine strategies becomes more attainable.

An intact innate immune response was shown to be crucial in containing a primary *B. pertussis* infection in mice. Infectious agents have evolved multiple strategies to interfere with the development of host immunity at several levels. As DC have a key function in connecting innate and adaptive immune responses, the suppression of DC maturation, migration and antigen-presentation as well as the inhibition of DC mediated T cell activation is an important immunosubversion strategy evolved by pathogens to facilitate their survival in the host. Tracheal cytotoxin (TCT), a member of the muramyl peptide family, has previously been shown to be an important component of *B. pertussis* immune evasion strategies. *B. pertussis* paralyses the ciliary clearance function of the respiratory tract through the release of TCT. The NO mediated ciliostasis induced by TCT facilitates the survival and replication of *B. pertussis* in the respiratory tract (Cookson *et al.*, 1989a). TCT

has also been reported to be toxic for human neutrophils, which may contribute to *B. pertussis* survival in the respiratory tract (Cundell *et al.*, 1994).

Several studies have reported synergy between another member of the muramyl peptide family, MDP and LPS for the induction of a range of cytokines from human cell lines or human blood (Traub *et al.*, 2004; Wang *et al.*, 2001; Wolfert *et al.*, 2002; Yang *et al.*, 2001). TCT synergises with LPS to induce IL-1α, iNOS, NO production and inhibits DNA synthesis in hamster tracheal epithelial (HTE) cells (Flak *et al.*, 2000). Consistent with this synergistic interaction reported for TCT with LPS in HTE cells, the present study found that TCT synergises with LPS to induce IL-1β, IL-10 and MIP-1α by DC. Furthermore, TCT was found to synergise with other TLR agonists in the production of cytokines by DC; TCT synergises with the TLR9 ligand, CpG for IL-β, IL-12p70, IL-12p40 and IL-10 production from murine DC, and synergised with Pam-3Csk for IL-1β production. However, the interaction between TCT and DC was found to inhibit LPS-induced MHC class II upregulation on the DC. Antigen processing and presentation plays a critical role in the induction of adaptive immune responses. Central to this process is the MHC-associated presentation of processed antigens by the DC to the T cell. Recent reports have shown that several types of bacteria, including *mycobacteria, E. coli, Yersinia enterocolitica* and *B. pertussis* can decrease MHC class II expression on infected cells, inhibiting antigen processing and presentation to promote their survival (De Lerma Barbaro *et al.*, 1999; Noss *et al.*, 2000; Noss *et al.*, 2001; Shumilla *et al.*, 2004; Wojciechowski *et al.*, 1999; Zhong *et al.*, 1999). *B. pertussis* infection of human monocytes reduced cell surface expression of HLA-DR, without altering total cellular levels (Shumilla *et al.*, 2004). Using a PT mutant strain of *B. pertussis*, the authors identified that PT partially accounts for the decrease in cell surface expression of

HLA-DR on monocytes (Shumilla *et al.*, 2004). The present study demonstrated that TCT is capable of inhibiting LPS-induced MHC class II expression on DC, which resulted in an attenuation of the adaptive immune response.

Several studies have documented the ability of bacterial cell walls (BCWs) to exert immunosuppressive effects *in vivo* (Chen *et al.*, 2002; Regan *et al.*, 1988; Wahl *et al.*, 1988). Infection of human monocytes with *B. pertussis* inhibited their ability to stimulate tetanus toxoid-specific $CD4^+$ T cells (Boschwitz *et al.*, 1997). Mutant strains deficient in FHA and CyaA were incapable of inhibiting monocyte induced tetanus toxoid-specific T cell proliferation, suggesting that these virulence factors were essential for this inhibitory effect (Boschwitz *et al.*, 1997). The role of TCT was not examined in the study by Broschwitz and colleagues, but observations in the present study indicate that TCT has potent immunosuppressive activities on antigen presentation *in vivo*. TCT was shown to impair the antigen-specific T cell cytokine and proliferation response from mice immunised with the Th2 adjuvant, alum. Similarly, TCT suppressed antigen-specific cytokine production by T cells from mice immunised with a foreign antigen in the presence of LT, an adjuvant known to induce a mixed Th1/Th2 response. Therefore, TCT is capable of suppressing both $CD4^+$ Th1 and Th2 responses generated by immunisation with a foreign antigen with an adjuvant *in vivo*.

It has been reported that anthrax lethal toxin, a major virulence factor of *Bacillus anthracis*, can exert inhibitory effects on DC, ultimately resulting in an impaired adaptive immune response (Agrawal *et al.*, 2003). Similarly, *B. pertussis* may subvert host defence strategies by targeting the DC. Adoptive transfer studies showed that pre-treatment of DC with TCT completely inhibited antigen-specific IFN-γ and IL-10 production induced by injection of DC pulsed with KLH in the

presence of the TLR2 agonist, Pam-3Csk. Furthermore, a profound impairment of KLH-specific T cell proliferation by lymph node cells was observed in mice injected with DC pulsed with antigen and Pam-3Csk in the presence of TCT compared with that seen in mice that received DC pulsed with antigen and Pam-3Csk alone. This suggests that the interaction between TCT and DC results in the profound impairment of DC antigen presenting function *in vivo*. This observation gives us a new insight into *B. pertussis* pathogenesis; namely, that *B. pertussis* has evolved intricate mechanisms that impair DC function and the subsequent immune response. An understanding of the mechanism that TCT employs to modulate immune responses may in future help in the design of new therapy for controlling T cell mediated autoimmune and inflammatory diseases.

The results of this investigation have provided further evidence of the evolutionary benefit to pathogens in targeting the innate host defence system as a means of prolonging survival. A better understanding of the innate immune response and the pivotal role played by DC and the TLR family may facilitate the development of new therapeutic approaches to bacterial diseases. An appreciation of the role of the innate immune response not only helps to further our understanding of the pathogenesis of infectious diseases, but may also provide exciting new strategies for augmenting vaccine-induced immunity and may help to design new immunosuppressive therapies. Our growing insight into the innate immune response means that only now, are we beginning to fully understand the underlying mechanisms behind the success of the 'crude' yet effective Pw introduced nearly a century ago. At present, the knowledge gained from genome sequencing of pathogens such as *B. pertussis*, along with the ever expanding wealth of information about the

innate as well as adaptive immune responses increases our optimism for the future of rational design of new vaccines and immunotherapetics.

Chapter 7

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7.0 REFERENCES

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