Chlamydia pneumoniae and airways inflammation:

An investigation of the host cell-pathogen relationship

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ABSTRACT

C. pneumoniae is an obligate intracellular bacterium that has been implicated in the pathogenesis of chronic airways diseases such as asthma and COPD. *C. pneumoniae* undergoes a biphasic developmental cycle, alternating between infectious and non-infectious forms. However, it may also enter into a persistent state whereby it undergoes limited growth and division. It is this persistent state which may be of particular importance in the development of chronic airway inflammation and disease. A variety of cells are susceptible to *C. pneumoniae* but the relationship between *C. pneumoniae* and host cells with relevance to airways inflammation is not well described. The current studies were undertaken to investigate the host-cell pathogen relationship, with the aim of measuring basic immune responses and how these responses may allow persistent infection to develop. Utilising a combination of flow cytometry and ELISA's I have examined cytokine and surface molecule expression of cells in response to *C. pneumoniae* exposure. Airway epithelial cells were shown to respond to *C. pneumoniae* stimulation by increasing IL-8 and IL-6, cytokines involved in the recruitment and activation of inflammatory cells to the primary site of infection. My studies also show that monocytes respond to *C. pneumoniae* by increasing cytokine production. Increased concentrations of *C. pneumoniae* significantly increased IL-10 but decreased IL-12 monocyte expression, possibly causing an imbalance between Th2 and Th1 responses. Expression of surface molecules indicative of cellular activation, were increased by *C. pneumoniae* on monocytes, neutrophils and to a lesser extent on lymphocytes. In addition, monocytes reduced costimulatory molecule expression which may lead to diminished T cell activation, failure to clear infection and promote the development of persistent *C. pneumoniae* infection. Theses studies show that *C. pneumoniae* modulates a range of basic immune responses of the host cell. Costimulatory molecule expression by monocytes may play a role in determining whether primary *C. pneumoniae* infection is cleared and this coupled with inadequate T cell activation may lead to persistent infection which is associated with chronic respiratory diseases such as COPD and asthma.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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The blossoms cannot tell what becomes of its fragrance as it drifts away,
Just as no person can tell what becomes of their influence as they continue through life.

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PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

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PRESENTATIONS

TR McNamara, GL Hodge, R Scicchitano, PN Reynolds, MD Holmes. *C. pneumoniae* reduces monocyte costimulation of lymphocytes: A basis for persistent infection? Australasian Flow Cytometry Group Annual Scientific Conference (2004), Adelaide, South Australia, Australia. Oral Presentation.

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TR McNamara, GL Hodge, R Scicchitano, MD Holmes. *C. pneumoniae* alters CD80 expression in monocytes: Implications in asthma. European Respiratory Society Annual Scientific Congress (2002), Stockholm, Sweden. Poster Discussion.

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

7-Aminoactinomycin D	7-AAD
Activator protein-1	AP-1
Base pairs	bp
Chlamydia pecorum	*C. pecorum*
Chlamydia pneumoniae	*C. pneumoniae*
Chlamydia psittaci	*C. psittaci*
Chlamydia trachomatis	*C. trachomatis*
Cluster differentiation	CD (eg CD14)
Chronic Obstructive Pulmonary Disease	COPD
Degrees Celsius	°C
Deoxyribonucleic acid	DNA
Dulbecco's Modified Eagles Medium	DMEM
Elementary body	EB
Escherichia coli	*E. coli*
Fluorescein isothiocyanate	FITC
Foetal Calf Serum	FCS
Granulocyte Macrophage Colony Stimulating Factor	GM-CSF
Heat shock protein	HSP
Hours	hrs
Immunoglobulin	Ig
Inclusion Forming Units	ifu
Interferon	IFN
Interleukin	IL

Ionomycin	ION
Kilobase	kb
Keratinocyte Serum Free Medium	K-SFM
Lipopolysaccharide	LPS
Major histocompatibility complex	MHC
Major Outer Membrane Protein	MOMP
Mean fluorescent intensity	MFI
Micrograms	µg
Micro-immunofluorescent test	MIF
Microlitres	µl
Minutes	mins
Milligrams	mg
Millilitres	ml
Monocyte Chemotactic Factor	MCP
Macrophage Inflammatory Protein	MIP
Natural killer	NK
Nuclear factor of activated T cells	NF-AT
Nuclear factor kappa B	NFκB
Outer membrane protein gene	ompA
Peripheral blood mononuclear cell	PBMC
Phorbol 12-myristate 13-acetate	PMA
Phosphate Buffered Saline	PBS
Phycoerythrin	PE
Phytohemmagglutinin	PHA
Polymerase Chain Reaction	PCR
Reticulate body	RB
Revolutions per minute	rpm

Ribonucleic Acid	RNA
Sucrose Phosphate Glutamine Buffer	SPG
T cell receptor	TCR
T helper	Th
Tumour Necrosis Factor	TNF
Uninfected cell lysate	UCL

CHAPTER 1

INTRODUCTION

Chlamydia pneumoniae (C. pneumoniae) is a common human pathogen and the primary site of infection is the respiratory tract. This unique intracellular bacterium was officially designated as a distinct entity within the Chlamydia genus in 1989 by Thomas Grayston and colleagues (1). Seroepidemiological studies indicate that on average 50% of the adult population have a positive response to *C. pneumoniae* and it is predicted that 100% of the population will be infected at least once during their lifetime (2,3). Acute *C. pneumoniae* infection causes approximately 10% of community acquired pneumonia and 5% of bronchitis (4-6). Early research has focussed on bacteriology and characterisation of the organism itself. The unique life cycle of Chlamydia has enabled this bacteria to develop mechanisms of host immune system evasion and the development of chronic infection (7,8). Persistent infection with *C. pneumoniae* has been associated with chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease, which are characterised by significant inflammation of the airways. Chronic persistent infection is often asymptomatic and therefore may go unrecognised. Unresolved subclinical infection may lead to the eventual development of chronic lung inflammation and disease. This ongoing subclinical infection may be a significant factor in the perpetuation of airways inflammation and disease.

The impact of *C. pneumoniae* infection on the pathogenesis of chronic lung disease is not fully understood. In order to understand the influence of *C. pneumoniae* infection on the pathogenesis of chronic respiratory disease, the relationship between the host cell and pathogen must be elucidated. Research investigating the response of cells involved in lung defence mechanisms to *C. pneumoniae*, would help us to determine the significance of infection in the development of chronic inflammation in the airways.

With these issues in mind, the aim of the studies documented here was to investigate the relationship between host and pathogen. The studies focus on cells within the lungs, which are susceptible to *C. pneumoniae* infection and measure the outcomes in terms of inflammatory mediator release and modulation of cell surface antigen expression.

The Chlamydia Genus

The Chlamydia genus consists of four species including *C. trachomatis*, *C. psittaci*, *C. pneumoniae* and the most recent member, *C. pecorum*. *C. trachomatis* can be divided into three biologically distinct variants (biovars) mouse, trachoma and lymphogranuloma venereum (LGV). The trachoma and LGV biovars are the most widely studied and can be further divided into two and three serologically distinct variants (serovars) respectively. *C. trachomatis* is primarily a human pathogen and is responsible for the blinding eye condition, trachoma. It is also known for its association and causal role in sexually transmitted disease. It is the most common sexually transmitted pathogen causing pelvic inflammatory disease, cervicitis in females and epididymitis in males and urethritis in both sexes (9). *C. psittaci* predominantly infects birds causing psittacosis or ornithosis but also causes abortion of pregnancy in cattle (10,11). After contact with diseased animals, human infection can occur and causes psittacosis, which manifests as pneumonia. *C. pecorum* is a pathogen of cattle and ruminants affecting the central nervous system, respiratory and digestive systems (12). It is not known to infect humans. *C. pneumoniae* is a predominantly a human pathogen, although it is now also known to infect koalas and various reptiles (13,14). In humans, *C. pneumoniae* infection is associated with a variety of inflammatory disorders of the respiratory tract. It has also been linked to other conditions such as atherosclerosis, arthritis and multiple sclerosis (15-19).

Chlamydial genetics/genome/biochemistry

The *C. pneumoniae* genome is approximately 1.2 x 10^6 nucleotide pairs which is slightly larger than that for *C. trachomatis* (20). DNA homology between *C. pneumoniae* and *C. trachomatis* is low, with sequence homology ≤10% which is consistent with DNA relatedness between other chlamydial species (21). However, up to 80% of predicted coding sequences within the *C. pneumoniae* genome could be assigned a function based on coding sequences determined in *C. trachomatis*, demonstrating a high level of functional conservation between the two species (22). A number of genes found in *C. trachomatis* are not represented in the *C. pneumoniae* genome and vice versa. The tryptophan operon consists of a number of genes required for the biosynthesis of tryptophan. In comparison to *C. psittaci*, in which the tryptophan operon is complete, in the *C. trachomatis* genome, only some of the genes involved in this pathway are represented (23). This operon is completely missing from the *C. pneumoniae* genome, suggesting that *C. pneumoniae* is completely dependent on the availability of tryptophan from host cell nutrient pools (22,23). Genes located either side of the *C. trachomatis* tryptophan operon are also absent in the *C. pneumoniae* genome and include genes coding for proteins related to phospholipase-D and a family of phospholipid synthases (20,22). Most of the genes missing from the *C. pneumoniae* genome in comparison to *C. trachomatis* are classified as hypothetical proteins, sequences that are likely to code for proteins, but are yet to be identified (22).

Although the majority of the extra coding regions in the *C. pneumoniae* genome have not been assigned a function, it is suggested that they contribute to the diversity of *C. pneumoniae* in its biology including infectivity, survival and increased range of host cells susceptible to infection (22). Some of the increased coding regions within the *C. pneumoniae* genome represent a family of chlamydial polymorphic membrane proteins (Pmp) which is expanded from 9 Pmp's in the *C. trachomatis* genome to 21 in *C. pneumoniae* (22). Although the function of these proteins remains unknown, they are predicted to be localised to the outer

membrane of chlamydial organisms and thought to play a role in host-pathogen interactions (24).

Enzymes and counterparts required for DNA synthesis, repair, transcription and translation are well represented in the *C. pneumoniae* genome along with DNA helicases (22). The basic mechanisms required for regulating transcription are conserved within the Chlamydial genus with alternative RNA polymerase subunits and regulatory factors found in both *C. pneumoniae* and *C. trachomatis* (20,22).

C. pneumoniae is dependent on host cells for its energy source and its genome contains genes encoding ATP translocases supporting this notion (22). It does however also contain genes for a membrane ATPase but whether it functions to produce ATP is yet to be determined. The genome also contains genes encoding various dehydrogenases required for aerobic respiration and electron transport chains (22). Genes representing major metabolic pathways are also identifiable within the *C. pneumoniae* genome. The glycolytic and tricarboxylic acid pathways are both represented, although are incomplete with genes missing for key enzymes (20,22). Also represented is a complete system for the synthesis and degradation of glycogen suggesting a role for the use of glucose and glucose derivatives in chlamydial metabolism.

Chlamydial Antigens

Major Outer Membrane Protein

In the absence of peptidoglycan in the cell wall of Chlamydia, the major outer membrane protein (MOMP) accounts for 60% of the outer chlamydial membrane. This protein is cysteine rich with conservation of the positioning of the 7 cysteine residues across chlamydial species (**Figure 1.1**) (25). Disulphide crosslinking of cysteine residues within the MOMP and other cell wall proteins, helps to maintain the cell wall structure. The chlamydial genome contains genes encoding disulphide bond isomerases that are most likely involved in the crosslinking process (20). MOMP is detected in the outer membrane between 12 and 18

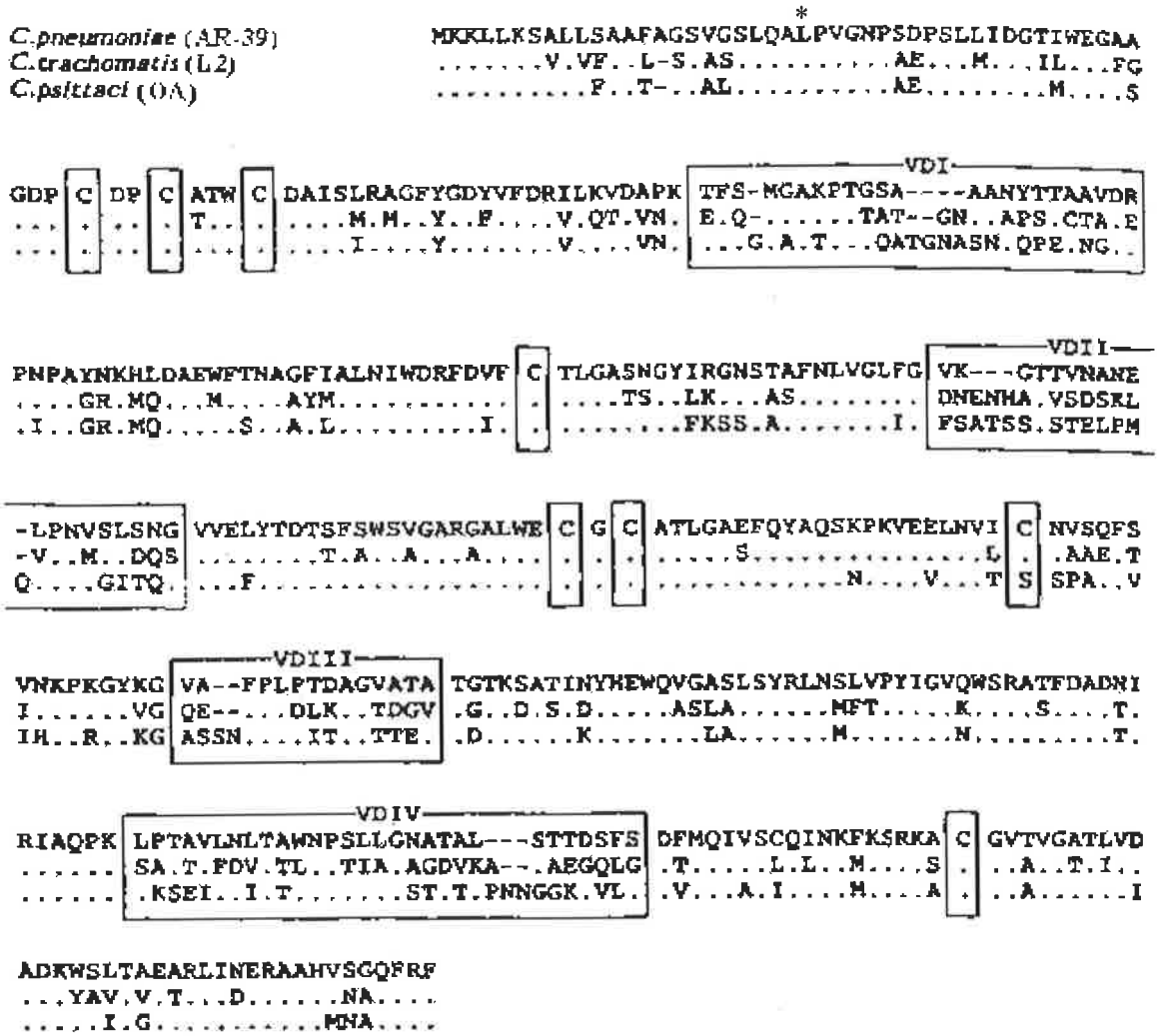


Figure 1.1: Amino acid sequence of *C. pneumoniae* MOMP and comparison with *C trachomatis* and *C. psittaci*.
C. pneumoniae isolate is AR-39, *C. trachomatis* isolate is serovar L2 and *C. psittaci* isolate is ovine abortion agent (OA). A dot represents an amino acid identical to that in the Chlamydia pneumoniae MOMP and a dash represents a gap in the sequence. Conserved cysteine residues and four variable domains (VD) are boxed. * Represents the N terminus of the mature MOMP sequence. Figure based on sequence published by Melgosa and colleagues (25)

hours after infection and remains present during the entire developmental cycle of Chlamydiae (26). The envelope protein and other outer membrane proteins are not detected until later in the cycle when reticulate bodies reorganise back into elementary bodies (discussed later) at around 30h post infection (26). Again these proteins contribute to the structural integrity of the outer membrane with extensive crosslinking to MOMP.

The MOMP gene, ompA, confers a mature peptide of 366 amino acids with a 23 amino acid signal peptide, finally giving rise to a protein of 40kDa (25,27). The ompA gene is conserved between *C. pneumoniae* isolates and contains four variable domains (25,27,28). The sequence of all four variable domains show little similarity with other chlamydial species but form loops between five highly conserved regions (25,27). Functional analysis of reconstituted MOMP suggest its involvement in formation of porin-like ion channels (29,30). MOMP appears to combine in trimeric structures within the outer membrane where they may play a role in movement of nutrients across the outer membrane structure such as nucleoside triphosphates (30).

Chlamydial heat shock protein-60

Heat shock proteins (HSPs) are generally a highly conserved group of proteins. They are expressed by cells under normal conditions but are significant induction is seen when cells are under stress such as exposure to increased temperature (31). Chlamydial HSP-60 is a homologue of GroEL in *Escherichia coli* (*E. coli*) and shares approximately 63% amino acid homology with its *E. coli* equivalent (32). However, *C. pneumoniae* HSP-60 demonstrates approximately 95% amino acid identity with other chlamydial HSPs (32). Although the function of HSPs are not completely understood it is thought that they play a role in ensuring correct conformational folding of proteins and the formation of protein complexes (33). Chlamydial HSP-60 may be associated with the outer membrane either at its periphery or anchored within the membrane (34). It has therefore been suggested that chlamydial HSP-60 may be involved in reorganisation of cysteine rich proteins within the elementary body outer

membrane, to aid the conversion of the bacteria to its intracellular form, the reticulate body (35).

Chlamydial lipopolysaccharide

One of the main constituents of the chlamydial outer membrane is endotoxin. Chlamydial lipopolysaccharide (LPS) is similar to most other gram negative endotoxins and consists of a polysaccharide portion covalently liked to the lipid A component. The polysaccharide component of chlamydial LPS consists of a pentasaccharide, which also incorporates a genus specific epitope (36). Structural studies using *C. trachomatis* have identified this unique epitope as a trisaccharide moiety (37,38). The lipid A portion of Gram negative bacteria is the most consistent portion of the structure of LPS. However, while most LPS molecules contain six fatty acid chains attached to the lipid A core, chlamydial LPS only contains five (36,39) Furthermore, the length of the fatty acid chains is longer than most other bacteria (39). As it is the lipid A portion of LPS which determines the potency of the molecule, it is suggested that the extended length of the fatty acid chains within the chlamydial LPS molecule accounts for the lack of potency in comparison to other Gram negative bacteria (40). In comparison to LPS derived from *Salmonella minnesota* or *Neisseria gonorrhoeae*, chlamydial LPS is 100 fold less potent in stimulating TNF-α production by whole blood (41). Activation and signalling pathways mediated via the LPS receptor, CD14 is also common to chlamydial derived LPS, but are less potent than other Gram negative endotoxins (41). There are four main ways in which cells react to LPS exposure: phagocytosis, proliferation, differentiation and release of cellular mediators and these activities are increased by LPS associating with LPS binding protein (36).

Chlamydial Developmental Cycle

The developmental cycle of Chlamydia is the main characteristic that sets it apart from most other bacteria. It is a unique intracellular bacteria that exists in two distinct developmental forms. The infectious form is termed an elementary body (EB) and exists as a small structure

of approximately 0.3μm. Although EB's are metabolically inert they do possess DNA, RNA, ribosomes and a central nucleoid structure (42). A trilaminar cytoplasmic membrane (**Figure 1.2**) and a second trilaminar membrane, the outer membrane, surrounds the electron dense core (26). The outer membrane is similar to the cell wall of Gram negative bacteria, which enables the EB to survive outside the host cell providing resistance to external factors. The rigidity of the membrane is aided by extensive crosslinking between membrane proteins as discussed earlier.

The second developmental form is termed a reticulate body (RB). It is the larger of the two forms and range in size from 0.8 to 1 μm. They are metabolically active and as a result contain approximately three times the amount of RNA as that of EB's (26,43). They posses enzymatic activity required for DNA, RNA and protein synthesis but do not have the enzymes required for ATP production and are thus dependent on the host cell for energy (44). The RB's are also dependent on and in competition with the infected host cell for precursors such as amino acids and nucleotides along with nutrients, vitamins and cofactors (7). Reduced disulphide crosslinking within the outer membrane of the RB gives rise to a structure less rigid than that seen in the EB, and renders it more permeable facilitating the transfer of host cell derived precursors and nutrients (7).

The EB is responsible for attachment and entry into the host cell (**Figure 1.3**). The means by which this process occurs is largely unknown but may involve the host cell cytoskeleton or a specific receptor-ligand interaction, as the net charge of both chlamydia and the host cell is negative. It has been suggested that the highly negative glycosaminoglycan molecule is involved in attachment of Chlamydia to host cells susceptible to infection. Recent studies show that both glycosaminoglycans on the surface of EB's and on the host cell surface are involved in the attachment of *C. pneumoniae* to bronchial epithelial (45). Entry into the host cell is suggested to be via phagocytosis or ingestion of chlamydia through clathrin coated pits

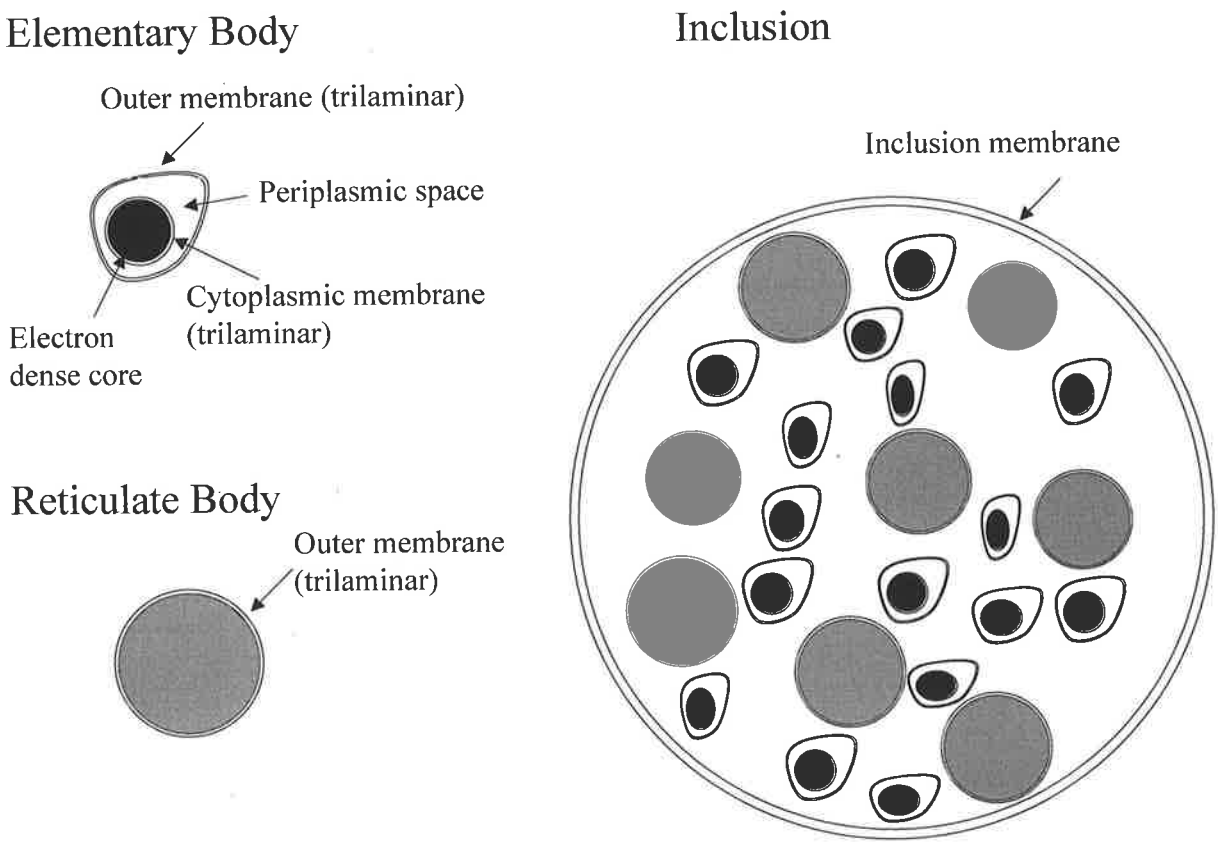


Figure 1.2: Schematic representation of *C. pneumoniae* EB, RB and inclusions.
Compiled from information and micrographs published by Schachter (26) and Grayston and colleagues (1).

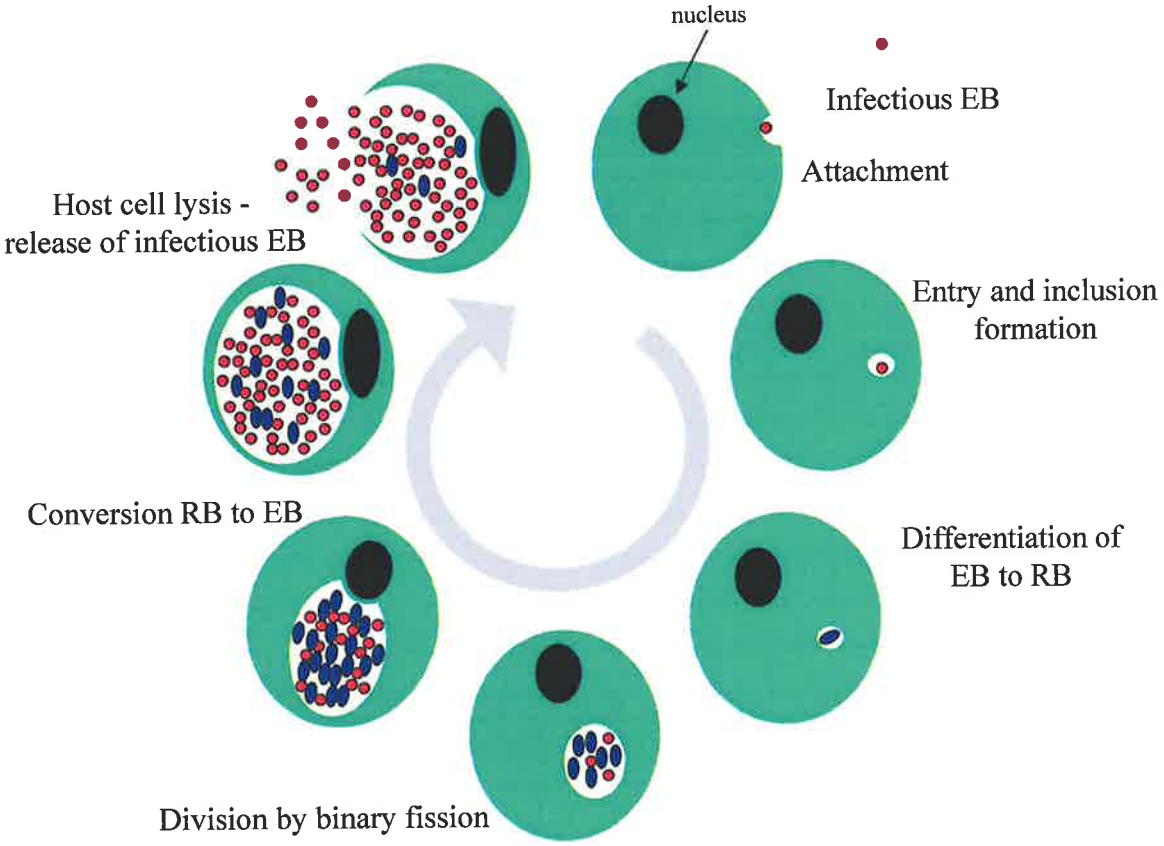


Figure 1.3: Schematic representation of the life cycle of Chlamydia.
The infectious EB attaches and enters into the host cell, followed by formation of the chlamydial inclusion and inhibition of phagolysosomal fusion. The EB then undergoes differentiation into an RB, which grows and divides by binary fission with a subsequent increase in inclusion size. The RB's undergo secondary differentiation back into EB's generating hundreds or thousands of infectious chlamydial EB's. The cycle is completed by host cell lysis and release of infectious EB allowing further infection of susceptible host cells. Figure based on that published by Beatty and colleagues with modifications (7).

(43). Attachment and entry into the host cell is also thought to be an energy dependent process and usually completed within 8 hours. Once inside the cell, the EB is enclosed within a membrane bound vesicle, termed an inclusion (**Figure 1.2**) where it enters into the next phase of development. In general, engulfment of pathogens into endosomes is followed by fusion with lysosomes, to form phagolysosomes, enabling breakdown of ingested particles. Chlamydiae however, inhibit phagolysosomal fusion thus allowing growth and development to progress (46). The mechanism for this inhibition is yet to be fully identified. However, when isolated chlamydial cell walls are ingested, fusion is inhibited (47). Ingestion of isolated RB's results in phagolysosome formation suggesting that the signal for inhibition of fusion, is not present on the surface of RB's (48). Inhibition of fusion allows the EB to undergo morphological changes and develop into the larger chlamydial form, the reticulate body (RB) which takes place over the next 6 to 10 hours after initial attachment. Alterations within the structure of the outer membrane decrease rigidity and increase permeability thus allowing uptake of ATP and nutrient transfer between host cell and chlamydial bodies. Reticulate bodies grow and divide by binary fission and EB's ultimately give rise to numerous RB's all within a single inclusion. The inclusion continues to expand until the RB's condense back into EB's which starts to occur 16-20 hours after initial infection (43). The timing of chlamydial development within a single inclusion is not synchronised and at this stage some RB's continue to divide and at any one time there may be a heterogeneous population of chlamydial bodies within the inclusion (26). The growth and division of RB's continues for up to 72 hours post infection. Several EB's may also be derived from a single RB and at the conclusion of the developmental cycle, a single inclusion may contain hundreds or thousands of EB's (26,43) ready for release from the host cell. Release of Chlamydiae from the infected cell occurs through lysis of the host cell or exocytosis allowing further infection of susceptible host cells (26,43,49). The lifecycle of Chlamydia from attachment to the host cell to release of new progeny is completed within 72 to 96 hours.

Persistence

The biphasic lifecycle of Chlamydia is not the only feature that has made this bacteria so unique. During the growth phase within the host cell, chlamydial organisms are able to develop a persistent infection whereby there is limited growth and division (**Figure 1.4**). Persistent infection is defined as a long term association between the bacteria and host cell where the bacteria is viable but unable to be cultured (7). During this state, Chlamydia infected tissues undergo morphological changes demonstrating fewer infected cells, less chlamydial inclusions per cell and less infectious progeny (49). Typical inclusions, as mentioned above, are tightly packed with both RB's and EB's of regular size and shape (50). In persistent infection inclusions ('altered inclusions')are smaller in size with decreased numbers of RB's and EB's. The RB's however, are larger than normal up to 2.5 μm in diameter (50). A third type of inclusion, referred to as aberrant, is smaller than normal inclusions with even less chlamydial bodies present. Bodies similar in size to the typical RB are present but there is no evidence of EB's (50,51).

The development of persistent infection is often governed by external factors and alteration in growth conditions such as nutrient deprivation, the presence of antibiotics or host cell derived factors. Chlamydial development can be delayed or interrupted at various points during the life cycle depending on the agent responsible for inducing persistent infection. It has been shown that absence of or lowered levels of cysteine delay the development of Chlamydia by interrupting the reorganisation of RB's to EB's (52) thereby inhibiting further progression of the chlamydial developmental cycle. This inhibition is reversible upon restoring levels of cysteine. Similarly, the deprivation of isoleucine in cell cultures of *C. psittaci* prevents not only chlamydial growth but also host cell growth (53) and is reversed on addition of isoleucine to culture media.

Numerous antibiotics are also able to inhibit the growth or differentiation of Chlamydia. Penicillin interrupts growth at two stages. It prevents binary fission and therefore division of

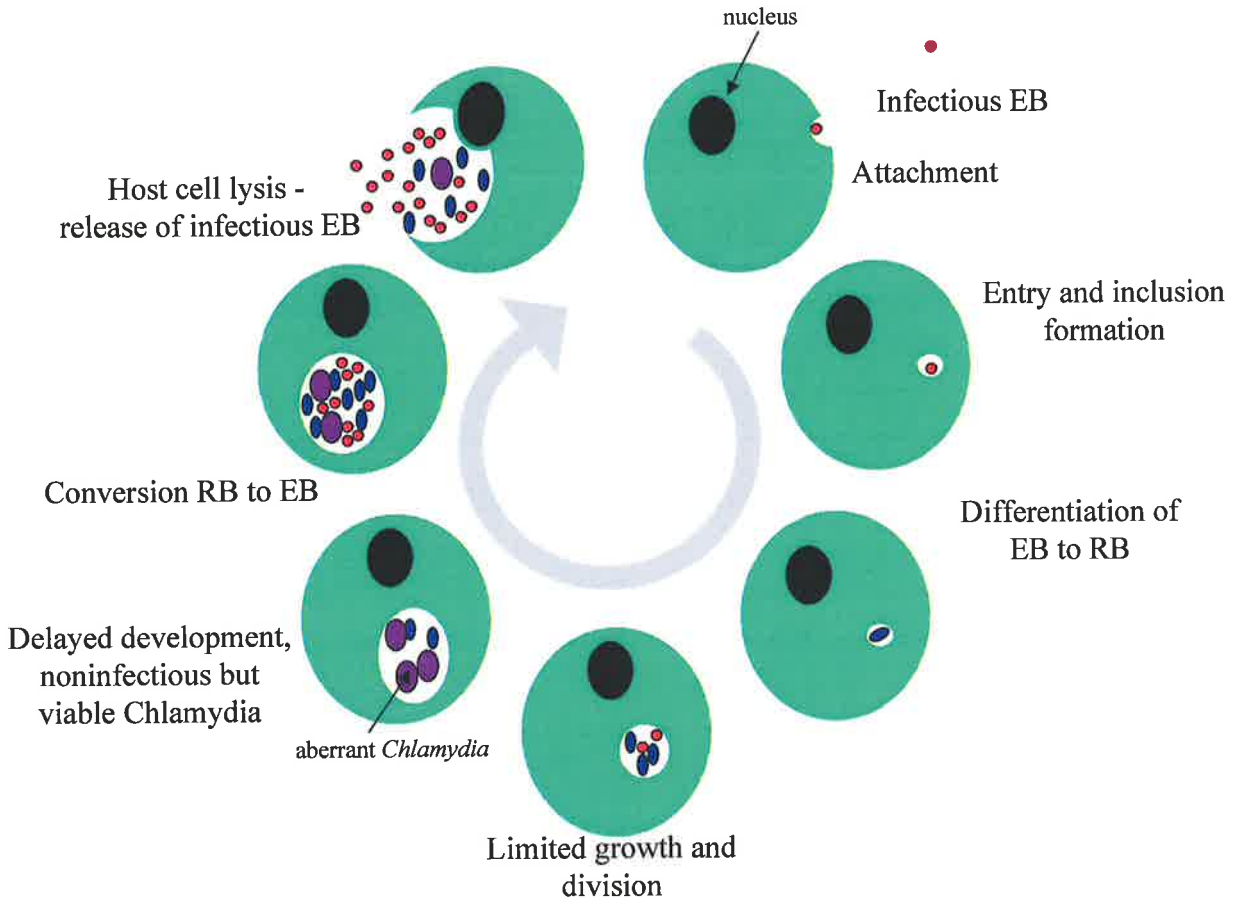


Figure 1.4: Schematic representation of altered or persistent chlamydial life cycle.
The infectious EB attaches to and enters into the host cell followed by formation of the chlamydial inclusion as in the regular chlamydial developmental cycle. The EB undergoes differentiation into an RB but undergoes limited growth and division. The inclusion is smaller than usual and Chlamydia undergoes delayed development with the formation of aberrant chlamydial bodies. Chlamydia are viable but non infectious with the absence of EB's. RB's eventually undergo secondary differentiation into EB's followed by release after host cell lysis but less infectious progeny are released than usual. Figure based on that published by Beatty and colleagues with modifications (7).

RB's and inhibits the differentiation of RB's to EB's (54). Ampicillin also disrupts the reorganisation of RB's to EB's (55). It has been proposed that the mechanisms by which penicillin and ampicillin alter chlamydial development is by decreasing the expression of chlamydial proteins rich in cysteine essential to the chlamydial membrane structure (55,56).

The effect of other antibiotics is dependent on the point at which they are added to cell cultures such as the case with chloramphenicol and chlortetracycline. The presence of these antibiotics early in the chlamydial life inhibits the differentiation of infectious EB's into RB's (57,58). Present later in the development, RB division is inhibited or secondary differentiation from RB to EB is blocked (57,58). Similarly, erythromycin also restricts the development of Chlamydia by inhibiting the transformation of RB's into EB's (59).

Some host cell derived factors such as cytokines are also able to alter the normal developmental cycle of Chlamydia. It was discovered that chlamydial infection results in increased degradation of tryptophan due to induction of the enzyme responsible for its catabolism, indoleamine 2, 3-deoxygenase (IDO) (60). Further research discovered that supernatants from chlamydial infected cells were capable of inducing IDO activity in a dose dependent manner suggesting the effects were brought about by the presence of host derived factors (60). It has also been shown that interferons inhibit chlamydial growth and inclusion formation is decreased proportional to the concentration of interferon (61-63). It is now known that interferon induced inhibition of chlamydial growth is due to increased activity of IDO and tryptophan breakdown (51,60,62,63). Chlamydial infection may initially induce host cell production of IFN-γ as a defence mechanism, inducing breakdown of tryptophan to kynurenine and N-formylkynerenine, and thereby reducing the availability of tryptophan, which is essential for chlamydial growth (49). However, unlike *C. psittaci*, which has the ability to utilise kynurenine as a precursor for tryptophan biosynthesis, *C. pneumoniae* does not possess the proteins required for the enzymatic conversion of kynurenine to anthrnilate

and eventual synthesis of tryptophan. Therefore induction of IDO activity by host cells may be a defence mechanism that promotes persistent chlamydial infection (23). It is also postulated that *C. pneumoniae* is more dependent on the host cell for tryptophan and is more susceptible to IFN-γ induction of persistent infection than *C. psittaci* (23,51,64). Persistent chlamydial infection induced by interferon as a result of tryptophan degradation, is reversed by removal of interferon or addition of exogenous tryptophan (61,63,65).

Interferons are however, not the only host derived factors capable of inducing persistent chlamydial infection via IDO activity and tryptophan breakdown. It has been shown that tumour necrosis factor inhibits the growth of *C. trachomatis* and synergises with interferon-γ (66,67). Similarly IL-1 has also been shown to synergise with interferon to induce IDO activity (62). The induction of IDO activity and tryptophan breakdown by TNF-α and IL-1, is also reversible by addition of tryptophan similar to the effects seen with interferon (62,66). As a result of decreased availability of tryptophan and induction of persistent chlamydial infection, cells infected with chlamydia show increased numbers of atypical inclusions with larger than normal RB's not dissimilar to that seen after antibiotic induced persistence (51,54).

During persistent infection, chlamydial antigen expression is altered. This may aid the organism in evading detection by the immune system but also results in failure to detect clinical infection. Chlamydial HSP-60 expression remains unaltered during persistent infection (65,68), but expression of other antigens such as MOMP, the 60kDa Outer Membrane Protein (ompA) or chlamydial lipopolysaccharide are changed. There is some evidence of upregulation of ompA (68,69) and MOMP (70) whilst others have reported significant decreases of ompA, LPS and MOMP in interferon induced persistence (56,65).

Chlamydia pneumoniae

The organism that we now know as *C. pneumoniae* was originally isolated from the conjunctiva of a Taiwanese child during a trachoma vaccine study in 1965, designated TW-183 (71). The second isolate was not obtained until 1983, designated AR-39, from a university student in Washington who presented with pharyngitis (3). It was from these two isolates that the strain name TWAR was derived.

Historically, TWAR was thought to be an unusual form of *C. psittaci*. Morphologically it closely resembled *C. psittaci*, with dense oval shaped inclusions and the absence of glycogen, unlike *C. trachomatis* inclusions which contain glycogen and vacuoles (71,72). However, classically defined infection with *C. psittaci* is associated with transmission to humans from an avian source. The lack of evidence for an avian source suggested that this new pathogen was a unique *C. psittaci* species with a human host (71,73). It was not until DNA homology and ultrastructural studies were performed that TWAR was noted to be a distinct entity. Based on restriction endonuclease analysis TWAR isolates were easily distinguished from both of the other Chlamydia species, trachomatis and psittaci (74). It was also shown that TWAR did not contain plasmid DNA, unlike the trachomatis isolates and the majority of psittaci isolates (74). Furthermore, all isolates tested in this study showed identical banding patterns with all restriction enzymes used.

DNA homology studies confirmed these findings that TWAR was distinct to other chlamydial species with ≤10% DNA homology to *C. trachomatis* and *C. psittaci* (21,74). This is consistent with DNA homology between trachomatis and psittaci, also ≤10% (21,75). Both restriction assays and DNA relatedness studies demonstrate that individual TWAR isolates are virtually identical with ≥94% sequence similarity confirming that TWAR isolates belong to the same serovar (21,74). In contrast, DNA homology within *C. psittaci* isolates is

variable, possibly contributing to the variety of hosts and diseases associated with this species (21).

Further investigation of TWAR morphology showed that TWAR was ultrastructurally different to *C. psittaci* and *C. trachomatis*. EB's of TWAR are typically pear shaped compared to the usual round appearance of *C. trachomatis* and *C. psittaci* (76). Furthermore, TWAR EB's have a large periplasmic space that is somewhat harder to define in the other chlamydial species. Both EB's and RB's of TWAR are surrounded by an immediate outer membrane and a plasma membrane, both of which exhibit a bilayer structure (76). The RB's of TWAR are round in shape with a much smaller periplasmic space, similar to other species of Chlamydia (76).

Complement fixation tests with antibodies raised against chlamydial group antigen, specific for the Chlamydia genus showed positive staining of TWAR inclusions and confirmed that TWAR was a member of the Chlamydia genus (71,72,77). However, specific monoclonal antibodies against TWAR only show reactivity with TWAR isolates and not *C. trachomatis* or *C. psittaci* strains providing further evidence of TWAR being distinct from these species (71,72,76). It was based on these studies of DNA homology, ultrastructural analysis and serology, that TWAR was officially designated as a new species of Chlamydia, the third in the Chlamydia genus, *C. pneumoniae* (1).

Epidemiology and Prevalence of Chlamydia pneumoniae

The most common means of diagnosis of *C. pneumoniae* infection is serology. The 'gold standard' used in the diagnosis of *C. pneumoniae* infection is the microimmunofluorescence test which detects *C. pneumoniae* specific serum antibodies, and is able to differentiate between chlamydial species (78,79). The commonly encountered problem with this test is that there is no consensus or universal definition of primary, acute, previous or chronic

C. pneumoniae infection and interpretation of results requires highly experienced laboratory personnel. Interpretation of prevalence also varies from study to study. Some reports of prevalence are based solely on the presence of IgG antibodies, with titres within the range of 1:16 to 1:256 (4). Other studies take into account IgM positivity with titres ≥ 1:8 (or IgG ≥ 1:16) as indicative of positive serology (80). Other investigators examine all three antibodies and define prevalence using titres of IgG ≥ 1:32, IgM ≥ 1:16 or IgA ≥ 1:32 (81). However, seropositivity is simply an indication of previous exposure to the pathogen and therefore not a true indicator of the prevalence of infection.

Acute infection may be described as current or recent infection and defined by a four-fold or greater change in titre of IgG, IgA or IgM (3,82,83). Other investigators describe acute infection as a four-fold increase in IgG or IgM titre (alone or together), an isolated IgG titre of ≥1:512 or an isolated IgM titre of ≥1:16, which is sometimes described as a primary or first infection (3,6,84-86).

Evidence of past infection is based on serological studies with specific antibodies to *C. pneumoniae* with past or pre-existing infection defined as IgG antibody titres of ≥1:16 or <1:512 (3,84,87-89). The definitions of *C. pneumoniae* infection however are not universal and some studies use systems based on cut off values and in these cases, previous infection is defined as IgG titres starting from as low as 1:8 - 64 at the lower end of the range extending up to 1:256 rather than 512 (86,90-92). However, diagnosis of past or pre-existing versus chronic infection is difficult to assess since some studies also define chronic infection with this same criteria (86,89,91). It has been suggested that the presence of *C. pneumoniae* specific IgA antibodies or secretory IgA (in sputum) is indicative of chronic infection (83,93,94) and therefore the absence of which along with the presence of serum IgG may be a more reliable marker for past infection (87).

With the suggestion that the presence of *C. pneumoniae* specific IgA antibodies is representative of chronic infection, the criteria used to diagnose chronic infection by serology is the presence of IgG titres ≥ 1:128 with concomitant IgA antibody titre of ≥ 1:40 (95). Although, some authors have used less stringent IgA titre levels starting at ≥ 1:8 or a higher level of ≥ 1:64 (82,92).

In summary, the most commonly used criteria to diagnose *C. pneumoniae* infection using the MIF test are shown in **Table 1.1**.

Infection	**Serology**
Acute, current or recent	Four-fold increase IgG, or IgM, or IgA Four-fold increase IgG and/or IgM IgG ≥ 1:512 IgM ≥ 1:16
Past, pre-existing	IgG ≥ 1:16 to 1:512 or ≥ 1:16 to 1:256
Chronic/pre-existing	Presence of IgA IgG ≥ 1:128 and IgA ≥ 1:40 or ≥ 1:64

Table 1.1: Level of chlamydial infection and corresponding serology.
Table compiled from references (3,82-85,88,96).

The majority of data reporting the population prevalence of *C. pneumoniae* is based on the evidence of past infection or the presence of pre-existing *C. pneumoniae* specific antibodies (3,90). In an early study, the prevalence of IgG specific antibodies to *C. pneumoniae* ranged greatly, from as low as 3% in Sierre Leone and up to 67% of people living in areas of Iran, with an overall average of 20% (80). In separate studies, the prevalence of IgG antibodies is reported to be even higher in countries such as Ireland and Israel at 70% and 74% respectively (97,98). Overall, the population prevalence in people free of clinically recognisable respiratory infection is estimated to be 50% with the expectation that everyone will be infected at least once with the organism and a second infection later in life is a common occurrence (4).

Numerous studies have indicated that the prevalence of *C. pneumoniae* infection increases with age (3,90,99,100). The frequency of infection is low in children and increases rapidly when children are of school age. The incidence of infection then increases rapidly during the teenage years until adulthood and remains at a steady state during the adult years. *C. pneumoniae* infection is more prevalent in males than females (71,84,101,102).

Clinical relevance

A number of respiratory illnesses in both the upper and lower respiratory tract, have been shown to be associated with *C. pneumoniae* infection. Acute infection is associated with pneumonia, pharyngitis, sinusitis, bronchitis and exacerbations of disorders of a more chronic nature including chronic obstructive pulmonary disease and asthma (71,83,85,99,103-105).

Community Acquired Pneumonia

An epidemic of pneumonia in Finland in two separate communities, provided the first evidence that TWAR was a respiratory pathogen (73). This was followed by numerous isolations during a two and a half year study of university students with acute respiratory disease (71). Infection was predominantly associated with students suffering from pneumonia with 12% of pneumonia patients having serology demonstrative of recent infection with *C. pneumoniae*. In addition to this, the organism was isolated from over half of the pneumonia cases. It has since been reported that community acquired pneumonia is one of the most frequent clinical manifestations of *C. pneumoniae* infection (90). Seroprevalence of acute *C. pneumoniae* infection in community acquired pneumonia patients ranges from 1-26% (71,83,84,102,106,107). Based on polymerase chain reaction detection of *C. pneumoniae* infection, incidence of infection in patients with community acquired pneumoniae ranges from approximately 5 to 38% (95,106,108). In children presenting with community acquired pneumoniae, the organism could be isolated from 19% of patients (109). Of these children,

27% showed evidence of previous infection and 27% showed evidence of acute infection as determined by serology.

Pharyngitis

C. pneumoniae can cause upper respiratory tract infections. In patients with chronic pharyngitis, *C. pneumoniae* is localised to the mucous membrane of the pharynx and could be cultured in a third of patients (103). Although this study was relatively small, a large proportion (75%) of the patients had serological evidence of acute infection. The elevations in antibody titres were representative of ongoing *C. pneumoniae* infection suggesting that *C. pneumoniae* is a causative agent in chronic pharyngitis (103). A slightly larger investigative study of 24 patients with presenting with symptoms of pharyngitis, showed that *C. pneumoniae* could be isolated from approximately 58% of nasopharyngeal specimens from patients presenting with symptoms of pharyngitis (85). Similar to the study by Falck and colleagues (103) serology and continued isolation of the organism suggests that *C. pneumoniae* infection persists for an extended period of time after the initial infection (85). An earlier study suggested that the incidence of *C. pneumoniae* infection in patients diagnosed with pharyngitis is much less with only 1% of patients showing serological evidence of recent or acute infection (71).

Bronchitis

Bronchitis is an infection of the lower airways and has been linked to infection with *C. pneumoniae*. In a study of patients experiencing acute wheezing illnesses, it was shown that primary exposure to *C. pneumoniae* could result in subsequent development of chronic bronchitis (6). In stable chronic bronchitis, the prevalence of *C. pneumoniae* infection ranges from 38 to 43% based on detection by polymerase chain reaction (104). In children, with chronic bronchitis or pneumonia the incidence is reported to be slightly higher at 52% (110). Further research also demonstrated that the frequency of exacerbations of chronic bronchitis

are increased in patients with positive polymerase chain reaction results for *C. pneumoniae* (104).

Chronic obstructive pulmonary disease

The association between *C. pneumoniae* infection and chronic obstructive pulmonary disease (COPD) is an active area of research. A number of studies have investigated the relationship between acute exacerbations of COPD and the incidence of *C. pneumoniae* infection. In comparison to control subjects, the levels of *C. pneumoniae* specific IgG antibodies is significantly higher in COPD patients compared to control patient groups (86,89,91). However, there are some studies that do not find any differences between control subjects and COPD patients (105). The overall incidence of infection based on the presence of *C. pneumoniae* specific antibodies is relatively high amongst COPD patients experiencing acute exacerbations ranging from approximately 60 to 96% (81,86,91,93,95,111). This suggests that the majority of COPD patients have previously been infected with *C. pneumoniae*.

However, acute or current infection with *C. pneumoniae*, is reported to be much lower amongst COPD patients ranging from approximately 3 to 34% based on serological data (83,86,89,91,93,111). Furthermore, acute infection with *C. pneumoniae* is described in the literature to be associated with 4-11% of COPD exacerbations (82,86,105). The airways of COPD patients are often colonised by a number of potential pathogens but *C. pneumoniae* is shown to be the sole causative agent of acute exacerbations in up to 16% of cases (89,111). In stable COPD patients the prevalence of *C. pneumoniae* infection ranges up to 27% as detected by polymerase chain reaction (112,113). A variety of clinical samples are used for detection of *C. pneumoniae* by polymerase chain reaction including sputum, peripheral blood mononuclear cells, nasal aspirates and swab specimens and may play a role in determining the efficiency of detection.

As mentioned earlier, chronic infection with *C. pneumoniae* is difficult to differentiate with past or previous infection using the microimmunofluorescence test and the presence of serum antibodies. However, it has been suggested that the criteria used to describe chronic *C. pneumoniae* infection is defined as a titre of IgG ≥ 128 and concomitant IgA ≥ 40, although some authors have used a higher IgA titre of ≥ 64 (82,95,114). Using the latter definition of chronic *C. pneumoniae* infection, 33.3% of COPD patients are demonstrated to be positive for chronic infection (82), which is significantly higher than corresponding healthy control patients. Not surprisingly, using the lower range for IgA titres, ≥ 40, Von Hertzen and colleagues demonstrated that a higher percentage of COPD patients (54%) are shown to fulfil the serologic criteria for chronic *C. pneumoniae* infection (83). A later study by the same group showed that 46% of patients with mild COPD and an even higher percentage, 71% of those with severe COPD are positive for chronic *C. pneumoniae* infection (95). This same trend with severity of disease was also confirmed by data measuring soluble IgA and detection of *C. pneumoniae* by polymerase chain reaction in sputum samples from the same patient population. The authors were then able to further define chronic infection with *C. pneumoniae* by positive results in two out of the three tests.

In acute exacerbation of COPD a study investigating causative agents, particularly *C. pneumoniae*, found no difference in the prevalence of chronic *C. pneumoniae* infection status between COPD patients and control patients (89). The study design used only persistently elevated IgA antibody titres as a measure of chronic infection which may explain the discrepancies with earlier studies. However, the authors did suggest that IgA antibody titres be evaluated with other *C. pneumoniae* specific immunoglobulins as suggested by earlier publications. Another study of patients with acute exacerbations of COPD found a similar prevalence with healthy patients using only IgG titres as a marker of chronic (or pre-existing) infection (111). However, the same study found elevated IgA antibody titres in the majority of patients suggested to have had reinfection with *C. pneumoniae*.

In summary, the majority of patients diagnosed with COPD have serological evidence of past infection with *C. pneumoniae*. In addition, the percentage of COPD patients with serology indicative of chronic infection with *C. pneumoniae* is suggested to be higher than those with serology demonstrative of acute infection. Despite the overwhelming wealth of evidence supporting a role of *C. pneumoniae* infection, particularly chronic infection, in the pathogenesis of COPD a number of questions remain. Whether *C. pneumoniae* infection predisposes individuals to the development of COPD, or whether reduced defences increase susceptibility to *C. pneumoniae* infection leading to the development of persistent infection, are areas that require further research. In addition, the contribution of chronic *C. pneumoniae* infection to increased inflammation and increased exacerbations of disease are also key aspects of *C. pneumoniae* infection that need to be investigated.

Asthma

One of the earliest indications that *C. pneumoniae* infection was associated with asthma came from a study investigating respiratory tract illness caused by *C. pneumoniae* (99). This study showed that the prevalence of wheezing was increased with increased *C. pneumoniae* antibody titre. Furthermore, some patients were diagnosed with asthmatic bronchitis after infection but not before. Overall, 47% of patients presenting with acute *C. pneumoniae* infection experienced symptoms of asthma including wheezing and bronchospasm. A further 21% were diagnosed with asthma after respiratory illness associated with *C. pneumoniae* infection and patients previously diagnosed with asthma experienced an increase in exacerbations. Acute infection with *C. pneumoniae* has been found in approximately 4-9% of asthmatics (6,96,115). A similar percentage have been associated with chronic asthma and acute exacerbations of asthma (6,116). In children, acute infection with *C. pneumoniae* is shown to be evident in 22% of subjects, using serologic criteria of a four-fold rise in IgG or IgM titres (88). Furthermore, *C. pneumoniae* could be isolated from significantly more asthmatic children enrolled in this study compared to healthy controls.

In children, a higher incidence of *C. pneumoniae* infection is associated with chronic respiratory disease including bronchitis and pneumonia (110). Reactive airways disease, defined as chronic mild asthma to chronic severe asthma is also associated with *C. pneumoniae* infection(88). Later investigations lead to the discovery of and increased prevalence of *C. pneumoniae* specific IgE antibodies in the serum of *C. pneumoniae* culture positive asthmatic children (117).

Despite the associations discussed above, there are a small number of studies that have not found an association between *C. pneumoniae* specific antibodies in adults and the prevalence of asthma exacerbations or a difference between control patients and asthmatics (92,118). It is also reported that in children experiencing acute exacerbations of asthma, no relationship exists with acute *C. pneumoniae* infection (119,120). Despite these findings, the study by Cook and colleagues found an association between previous exposure to *C. pneumoniae* and chronic asthma (92). Similarly, the investigation by Cunningham and colleagues provided evidence that chronic infection with *C. pneumoniae* was frequent in asthmatic children and associated with acute exacerbations (119). The study by Betsou and colleagues also found a significant difference between the prevalence of antibodies to chlamydial heat shock protein-10 in asthmatics compared to healthy controls (118). Similar findings have been observed with chlamydial heat shock protein-60 antibodies, the incidence of which is higher in asthmatics compared to control subjects (121).

Similar to studies investigating chronic *C. pneumoniae* infection and COPD, the relationship between persistent infection with *C. pneumoniae* is also important in asthma. The levels of *C. pneumoniae* specific IgA antibody titres are more often increased in asthmatics compared to control subjects (115,122). The levels of *C. pneumoniae* IgA antibodies are also increased in children experiencing increased episodes of asthma exacerbations (119). Elevated IgA

antibody titres may be indicative of chronic infection and are positively correlated to the severity of asthma (115). This data is also supported by evidence of high IgA titres in chronic asthma patients (6). Severe chronic asthmatic patients show serology evident of previous or chronic infection, using a definition of *C. pneumoniae* antibody titres of IgG 1:64 to 1:256 or IgA≥1:8, and is significantly increased compared to control subjects (92). Similar results are reported from asthmatics experiencing exacerbations demonstrating an increased prevalence of both IgG and IgA antibodies compared to control population, indicative of chronic infection or reinfection (96).

Asymptomatic infection

Although pneumonia and bronchitis are the most commonly associated clinical syndromes associated with *C. pneumoniae* infection, often the most likely result of infection may in fact be asymptomatic and may go unrecognised (3,5,90). The earliest report of asymptomatic *C. pneumoniae* infection was documented after accidental laboratory exposure to a clinical isolate of *C. pneumoniae* (123). Two cases were reported, one of which did not develop any immunological response as measured by the microimmunofluorescence test at the time of exposure or subsequent testing. However, isolation from a nasopharyngeal swab was positive for infection by culture at the time of exposure but later tested negative. The second case showed previous exposure before the accident by serology and later demonstrated a fourfold decrease in specific antibody titres. Isolation from this person remained positive at all time points tested. Neither subject presented with any symptoms of respiratory illness before, during or after the time of exposure to the organism. The authors concluded that although the time at which or how the individuals acquired the infections was not conclusive, they provided clear evidence that *C. pneumoniae* could cause asymptomatic infection (123). Since then, asymptomatic infection has been shown to occur in approximately 5% of adults and children by isolation from clinical specimens taken from the throat and nasopharynx (88,124). Using the microimmunofluorescence test, population prevalence in adults free of clinically recognisable respiratory disease, is documented to be much higher, ranging from 60% of a

population in Japan, 74% of an Israeli population and as much as 82% in Americans, all demonstrating specific antibodies to *C. pneumoniae* (98,125,126). In the same studies, the prevalence of acute *C. pneumoniae* infection in asymptomatic patients ranges from approximately 4 to 20% (98,125,126). Within the Israeli study the investigators also distinguished between past infection, defined as the presence of specific IgG titres ranging from ≥ 1:16 to 1:256, and persistent or chronic infection as antibody titres in the IgG fraction of 1:64 to 1:256 together with an IgA antibody titre of ≥ 1:20 (98). Using the criteria for chronic infection they reported that chronic or persistent infection occurred in 8% and 12% of teenagers and adults respectively. Unfortunately the studies mentioned earlier investigating the prevalence of *C. pneumoniae* infection in asymptomatic populations, (125,126) only demonstrated the incidence of acute infection and made no reference to chronic or persistent infection.

Host-Chlamydia pneumoniae interaction in the lungs

The association between *C. pneumoniae* infection and acute and chronic respiratory disorders is well recognised. In the lungs a number of cells are actively involved in the inflammatory process associated with such disorders. Some of these cells including airway epithelial cells and macrophages are also susceptible to *C. pneumoniae* infection (127-130). In the following sections I have sequentially reviewed many of the key mediators in inflammatory airways disease, then reviewed the evidence that *C. pneumoniae* may be playing a role in these processes.

Airway Epithelium

Physical properties of the airway epithelium

The airways of the respiratory system are lined by a specialised layer of cells. It consists of pseudostratified epithelium with each cell sitting on a basement membrane (**Figure 1.5**). These cells exhibit polarity with the apical surface covered with specialised hairlike processes called cilia. These cells are the most prominent in the proximal airways and are covered with

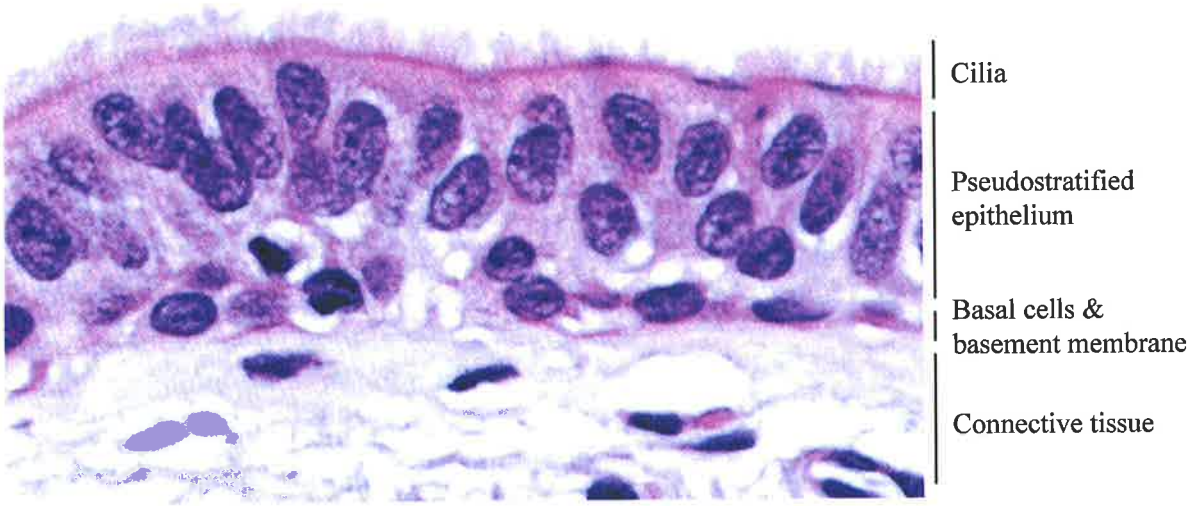


Figure 1.5: Representation of pseudostratified ciliated airway epithelium.
Each cell rests on the basement membrane, with cell nuclei at different levels, giving the impression that there are multiple layers of cells. Basal cells do not reach the luminal surface but are important in repair and regeneration of damaged epithelium. Note the polarity of cells with numerous cilia on the apical cell surface. Figure based on that of Burkitt and colleagues with modifications (131).

up to 300 cilia per cell (131). Beating of cilia requires energy and the placement of mitochondria in close proximity to the apex of the cell, provides energy for the motility of cilia and further demonstrates cellular polarity (132). Maintenance of polarity is aided by tight junctions between the cells and also provides the epithelial layer with strength maintaining an impermeable physical barrier (133). The epithelium also contains basal cells, thought to be important in repair and regeneration of damaged epithelium. Basal cells are positioned in the lower portion of the epithelial layer giving the epithelium its pseudostratified appearance (133,134). In the upper airways, the epithelial layer is also interspersed with goblet cells (**Figure 1.6**) making up 15-20% of the epithelial cell population, the number of which decreases in the lower airways (133). Goblet cells contain numerous secretory vesicles containing mucins which are released apically and responsible for giving mucous its properties of stickiness. The release of mucous from goblet cells aids in maintaining moisture of the lining, humidification during inspiration and defence of the lungs by trapping particulate matter and potential lung pathogens. The combination of rhythmic ciliary beating and mucous secretions form what is known as the mucociliary escalator. The beating movement of cilia contributes to lung defence by moving any foreign material trapped in the mucous toward the mouth where it is swallowed or expectorated. The airway epithelium is anchored to a connective tissue base of a basement membrane, lamina propria, which is separated from the submucosa, by a layer of smooth muscle (131). The airway epithelium does not have its own vascular supply and relies on diffusion of nutrients from underlying vascular tissue through the basement membrane and transport into the cell via the basolateral cell surface (132).

In asthma, there is significant damage to the epithelial cell layer. In comparison to the airways of normal subjects, the extent of damage to the epithelium in asthmatic patients is significantly increased (135). The damage to bronchial epithelium in diseased airways ranges from mild to severe. In some cases bronchial biopsies taken from asthmatics show that the

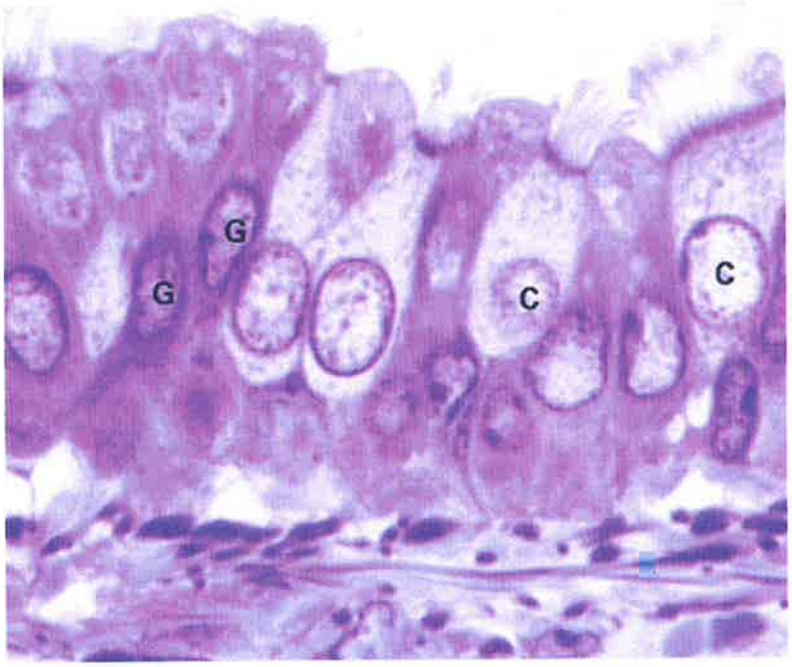


Figure 1.6: Pseudostratified airway epithelium with goblet cells.
G, Goblet cell; C, ciliated epithelial cell; Figure based on that of Burkitt and colleagues with modifications (131).

epithelial layer has been shed completely leaving a layer of basal cells, this process is referred to as desquamation (135,136). As a consequence of epithelial shedding, increased numbers of epithelial cells are found in bronchoalveolar lavage and sputum samples obtained from asthmatic airways (137,138).

The airway epithelium serves as the first line of defence for the respiratory system. One of its mechanisms of defending the lung against potential pathogens and noxious stimuli is to function as a physical barrier. One of the aspects in which it performs this function is with the aid of its specialised physical and morphological characteristics of the mucociliary escalator. In asthma, apart from shedding of the epithelial layer, efficiency of the mucociliary escalator is altered by disorganisation of cilia arrangement and the loss of cilia (136). The presence of abnormal cilia is also documented in patients with chronic bronchitis and bronchiectasis and is significantly increased compared to control subjects (139). Significant epithelial damage is also observed in children with chronic bronchitis (140). This study showed a loss of cilia in bronchial biopsies and a reduced rate of mucous transport.

Susceptibility of epithelial cells to C. pneumoniae infection

Despite the difficulties encountered by researchers in being able to consistently culture and maintain *C. pneumoniae*, research has shown that *C. pneumoniae* has the potential to infect a variety of cells both *in vivo* and *in vitro*. These cells include airway epithelial cells, epithelia from the urogenital tract, monocytes, macrophages, smooth muscle cells, endothelium, synoviocytes, fibroblasts and peripheral blood mononuclear cells (64,127,129,130,141,142).

The most commonly used cell line for *C. pneumoniae* culture is the HEp-2 cell line. HEp-2 cells are originally derived from a tracheal carcinoma. In comprehensive studies by different groups investigating the use of different cell lines and various *C. pneumoniae* isolates, it was determined that HEp-2 cells and NCI-H 292 cells, also a lung cancer cell line, were the most sensitive lines for propagation and maintenance of *C. pneumoniae* cultures (143-145). Prior

to this the other cell lines commonly used included HeLa 229 cells (cervical epithelium) due to its use in culture of *C. trachomatis*, and McCoy cells (murine fibroblast cell line).

Although HEp-2 cells have long been established as one of the most efficient cell lines in propagation of *C. pneumoniae*, they are not the only epithelial cells of the airways that have been shown to be susceptible to infection. Bronchial epithelial cells, the BEAS2B cell line and bronchial epithelium obtained by bronchoscopy are both susceptible to *C. pneumoniae* infection (127,128). In addition A549 cells, originally derived from an epithelial lung carcinoma, are also susceptible to *C. pneumoniae* infection.

Physical response of airway epithelial cells to C. pneumoniae infection

A small amount of research has been performed investigating the effect of *C. pneumoniae* infection on ciliary function. Bronchial epithelial cells obtained at bronchoscopy infected with *C. pneumoniae* show a decrease in ciliary beating which is completely abolished within 48 hours of infection (128). Further research showed that this effect was not due a decrease in available ATP (required for ciliary beating) and did not require host cell protein synthesis. As ultraviolet inactivation of *C. pneumoniae* failed to change the observed ciliostasis, it was suggested that the decrease in ciliary movement was not a direct effect of the infection but due to the presence of chlamydial antigen. In combination with this work, other research with murine models of mouse pneumonitis, inoculated with *C. pneumoniae* showed the presence of chlamydial inclusions in ciliated bronchial epithelial cells (146). These cells however, had lost the majority of cilia processes.

Cytokine expression by airway epithelial cells

Not only does the epithelial layer of the respiratory system act as a physical barrier, it is now known to actively participate in lung defence mechanisms. The respiratory epithelium is capable of responding to potential pathogens and playing a role in the initiation and regulation

of the immune response via synthesis and production of immunomodulators such as cytokines, and through recruitment, activation and direct interactions with inflammatory cells.

Numerous studies document the ability of airway epithelium to produce and secrete a variety of cytokines including TNF-α, interleukin (IL)-6, IL-8, IL-4, granulocyte macrophage colony stimulating factor (GM-CSF) and transforming growth factor (TGF) (147-152). These cytokines are important in a number of inflammatory processes including activation and priming of cells, recruitment of other inflammatory cells, growth and proliferation, switching of cytokine profile expression patterns and modulation of surface molecule expression (**Table 1.2**).

Airway epithelial cells are responsive to TNF-α or IL-1β stimulation, resulting in increased IL-8 expression (147,151,153). The combination of TNF-α and IL-1β often results in a synergistic increase in IL-8 (147,153). However, significant downregulation of IL-8 expression by bronchial epithelial cells occurs after treatment with IL-13 and IL-4 (154). Similar observations regarding IL-6 and GM-CSF expression by airway epithelial cells, which are upregulated by TNF-α and IL-1β (147). This data supports the notion that release of cytokines such as TNF-α and IL-4 from airway epithelial cells can further modulate expression of cytokines in an autocrine and paracrine fashion. Furthermore, TNF-α is also demonstrated to increase IL-4 expression, both of which decrease TGF-β expression (155,156).

The expression of a variety of cytokines is upregulated in response to pathogens such as viruses and bacteria. Exposure of primary airway epithelium or airway epithelial cell lines to respiratory syncitial virus (RSV) or rhinovirus increases TNF-α, IL-6, IL-8 and GM-CSF expression (149,153,157). Bacterial infection, such as with *Pseudomonas aeruginosa* and *Haemophilus influenzae*, also cause upregulation of TNF-α, IL-6 and IL-8 (150,158).

Cytokine	Cell source	Effects	Level in Airways
TNF-α	Monocyte/macrophage T cells Airway epithelium Mast cells Eosinophils	↑ GM-CSF, IL-8, ICAM-1 by airway epithelium ↑ ICAM-1, VCAM-1, endothelium ↑ IL-1 & MHC Class II by macrophages ↑ T cell & fibroblast proliferation ↑ BHR	↑ mRNA is bronchial biopsies of asthmatics ↑ mRNA in alveolar macrophages of asthmatics ↑ BAL fluid of asthmatics ↑ mRNA monocytes of asthmatics ↑ lymphocytes of COPD patients ↑ sputum of COPD patients
IL-1β IL-1α	Monocyte/macrophage T cells NK cells Neutrophils Eosinophils B cells	↑ by IL-1 and TNF in monocytes/macrophages ↑ T cell activation with IL-6 ↑ TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF, IFN-γ (variety of cells) ↑ ICAM-1, VCAM-1 on airway epithelium & endothelium ↑ fibroblast proliferation Chemotactic for neutrophils	↑ BAL of asthmatics ↑ mRNA in airway epithelium & alveolar macrophages of asthmatics ↑ in sputum from COPD patients ↑ eosinophilia & neutrophilia
IL-2	Activated T cells Airway epithelium Eosinophils	↑ Growth & differentiation of T cells, B cells, NK cells, monocyte/macrophage Chemotactic for eosinophils ↑ IFN-γ by NK cells ↑ IL-1 by monocytes ↑ phagocytic & cytotoxic activity of monocytes ↑ B cell immunoglobulin secretion	↑ in BAL of asthmatics ↑ GM-CSF production by peripheral blood mononuclear cells of asthmatics
IL-3	Activated T cells Mast cells	Growth factor for haematopoietic cells In conjunction with GMCSF, ↑ development of neutrophils, eosinophils, mast cells, monocytic cells, basophils	↑ in cells of BAL from asthmatics ↑ mRNA expression in biopsies from asthmatics
IL-4	Eosinophils Th2 cells	Promotes Th2 differentiation Inhibits Th1 differentiation ↑ cytotoxic activity of NK cells Activates B cells via MHC Class II & CD40 expression ↑ B cell immunoglobulin synthesis & switch to IgE synthesis ↑ MHC Class II expression by monocyte/macrophage ↓ TNF-α, IL-1, IL-12, IFN-γ, IL-8, MIP-α by macrophages ↑ mast cell growth in conjunction with IL-6 & G-CSF ↓ MMP expression by alveolar macrophages ↓ IL-8 expression by airway smooth muscle ↑ fibroblast activation & chemotaxis	↑ mRNA expression by BAL cells from asthmatics including CD4 & CD8 T cells, mast cells eosinophils
IL-5	Eosinophils T cells Mast cells	↑ development, maturation and activation of eosinophils Chemotactic for eosinophils ↑ Th2 development	↑ in sputum of asthmatics ↑ in BAL of symptomatic asthmatics ↑ in serum of asthmatics

IL-6	Monocyte/macrophage T cells Airway epithelial cells Fibroblasts B cells	↑ T cell & B cell differentiation ↓ TNF-α, IL-1 by alveolar macrophages ↑ IL-4 mediated IgE synthesis ↑ B cell immunoglobulin secretion	↑ alveolar macrophages of asthmatics ↑ BAL of CAP patients; ↑ in sputum of COPD patients
IL-8	Monocyte Airway epithelial cells Eosinophils Airway smooth muscle cells	Chemotactic for neutrophils Activates neutrophils to ↑ respiratory burst, intracellular calcium ↑ histamine release by basophils	↑ sputum of COPD patients ↑ BAL in chronic bronchitis & CAP patients ↑ mRNA alveolar macrophages of CAP patients ↑ mRNA bronchial epithelium of COPD patients
IL-9	T cells (Th2) Mast cells Eosinophils	↑ mast cell proliferation & differentiation ↑ T cell proliferation ↑ B cell immunoglobulin secretion	↑ mRNA in biopsies from asthmatics
IL-10	Monocyte/macrophage T cells (Th1 and Th2) Alveolar macrophage	Inhibits monocyte/macrophage function ↓ cytokine synthesis by monocyte/macrophage (TNF-α, IL-1, IL-6, IL-8, IL-12) ↓ MHC Class II, CD80, CD86 on monocytes ↓ IFN-γ, IL-2 by Th1 cells ↓ IL-4, IL-5 by Th2 cells Inhibits eosinophil survival ↑ B cell viability, proliferation & immunoglobulin secretion ↓ MMP, ↑ TIMP expression by tissue macrophages	Conflicting data ↓ sputum of asthmatics & COPD ↑ BAL of asthmatics ↔ in monocytes from asthmatics & normals
IL-11	Airway smooth muscle cells Fibroblasts Airway epithelial cells	↑ B cell differentiation ↓ IL-12 and TNF-α by monocyte/macrophage	↑ in severe asthma
IL-12	Monocyte/macrophage B cells Dendritic cells	↑ TNF-α secretion by T cells, NK cells ↑ T cell & NK cell cytotoxicity ↑ T cell proliferation Promotes Th1 differentiation ↓ IgE Inhibits Th2 differentiation	Conflicting data ↓ in peripheral blood of allergic asthmatics ↓ mRNA in biopsies of asthmatics ↑ BAL of asthmatics
IL-13	T cells	↓ TNF-α, IL-1β, MIP-1α secretion by alveolar macrophages ↓ IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12 by monocytes ↓ IL-8 by airway smooth muscle Inhibits Th1 development via ↓ IL-12 by monocytes ↑ eosinophil activation and survival	↑ mRNA in submucosa of asthmatic airways Levels correlated to eosinophils ↑ BHR
IFN-γ	Th1 cells NK cells	Inhibits Th2 cell activity ↓ IL-10 by monocytes ↓ IL-2 induced IL-8 by monocytes Activates alveolar macrophage/monocytes, epithelial & endothelial cells ↑ cytokine and adhesion molecule expression by airway epithelium ↑ MHC Class I & II macrophages & epithelial cells ↑ IL-12 by macrophages & dendritic cells ↓ IgE ↑ IL1 by monocytes	↓ in T cells of asthmatics Inhibits airway hyperresponsiveness Inhibits allergic eosinophilia

GM-CSF	Macrophages Airway epithelial cells T cells Eosinophils Fibroblasts	↑ TNF-α, IL-1 from monocytes ↑ eosinophil survival ↑ proliferation and differentiation of haematopoietic cells	↑ airway epithelium of asthmatics ↑ by monocytes of asthmatics
MIP-1α MIP-1β	Monocyte T cells Neutrophils	MIP-1α chemotactic for CD8 T cells & eosinophils MIP-1β chemotactic for CD4 T cells MIP-1α chemotactic for NK cells ↑ release of intracellular calcium	↑ MIP-1α in BAL fluid of asthmatics ↑ MIP-1α secretion increased by alveolar macrophages of asthmatics
MCP-1 MCP-2 MCP-3 MCP-4	Monocytes Airway epithelial cells Eosinophils	MCP1-3 chemotactic for monocytes MCP-2, MCP-3, MCP-4, chemotactic for eosinophils MCP1-4 chemotactic for T cells MCP-1 chemotactic for NK cells ↑ release of intracellular calcium MCP-1 ↑ IL-1, IL-6 by monocytes MCP-1 ↑ basophil histamine release	↑ MCP-1 in BAL fluid of asthmatics ↑ MCP-1 expression by airway epithelium of asthmatic & COPD patients
TGF-β	Monocyte/macrophage Lung fibroblasts Eosinophils Neutrophils Airway epithelium Airway smooth muscle cells Alveolar macrophages	Promotes differentiation of airway epithelium Chemotactic for fibroblasts, monocytes & mast cells ↑ TNF-α, IL-1& TGF-β by monocytes ↓ smooth muscle & IL-1 dependent lymphocyte proliferation	↑ mRNA in eosinophils of asthmatics and chronic bronchitis ↑ in epithelium of chronic bronchitis ↑ monocytes from COPD patients

Table 1.2: Cell source of cytokines and their relevance to chronic disease.
↑ = increased; ↓ = decreased; ↔ unaltered or no difference; TNF = tumour necrosis factor; IL = interleukin; IFN = interferon; GM-CSF = granulocyte macrophage colony stimulating factor; MIP = macrophage inflammatory protein; MCP = macrophage chemoattractant protein; TGF = transforming growth factor; NK = natural killer; BAL = bronchoalveolar lavage; COPD = chronic obstructive pulmonary disease; mRNA = messenger ribonucleic acid; MHC = major histocompatibility complex; Th = T helper cell; CAP = community acquired pneumonia. Compiled from references (159-162).

It is well documented that the expression of cytokines from airway epithelium from asthmatics is altered in comparison to that of healthy individuals. In particular, the expression of IL-1β, IL-6, IL-8, GM-CSF and TNF-α are increased from bronchial epithelium derived from symptomatic asthmatics compared to epithelium from control subjects (137,163-166). The levels of IL-6 and GM-CSF are elevated in bronchoalveolar lavage fluid from asthmatics when compared with controls or asymptomatic patients and is localised to cells including epithelial cells (137).

Primary bronchial epithelial cells from chronic obstructive pulmonary disease patients also exhibit altered cytokine expression. Compared to control subjects, IL-8 and TGF expression from bronchial epithelium, is significantly increased in chronic obstructive pulmonary disease patients (167-170). Similar results are observed for expression of monocyte chemotactic protein-1, where mRNA levels are significantly increased in bronchial epithelial cells of COPD patients compared to subjects without disease (168). Cytokines such as IL-8, MCP-1 and TGF are likely to play a role in recruitment of additional inflammatory cells to the site of inflammation in COPD. These mediators possess chemotactic activity for inflammatory cells such as neutrophils, monocytes, macrophages and mast cells (171-174). In addition the numbers of neutrophils, macrophages and mast cells are increased in the airways of COPD patients (175-177). TNF-α, which is reported to be elevated in sputum from COPD patients, is also able to subsequently enhance the already increased baseline level of IL-8 expression in bronchial epithelium (167,175). Levels of IL-8 are also reported to be increased in sputum obtained from COPD patients (175,178). Cigarette smoking is the major risk factor for developing COPD and it is documented that bronchial epithelial cells exposed to cigarette smoke exhibit increased IL-1β expression (179). Epithelial expression of TGF-β is increased and IL-8 protein measured in BAL fluid of chronic bronchitis patients, which could potentially be produced by epithelial cells, is also increased in comparison to control patients

(180,181). *Haemophilus influenzae* infection is often associated with chronic bronchitis and community acquired pneumoniae is reported to increase the expression of a number of cytokines from bronchial epithelium including TNF-α, IL-6 and IL-8 (148,182).

Airway epithelial cell cytokine response to C. pneumoniae

There is overwhelming evidence of the involvement of the airway epithelial cells in lung defence mechanisms, which include alteration to their cytokine expression profile. These cells are also susceptible to *C. pneumoniae* infection. In addition, there is also a strong indication of an association between *C. pneumoniae* infection and acute, as well as chronic lung diseases. Despite this evidence, the relationship between host (airway epithelium) and pathogen (*C. pneumoniae*) is not well defined. There are few papers in the literature investigating the response of airway epithelium to *C. pneumoniae* infection in terms of cytokine production. The first paper addressing this issue utilised a bronchial epithelial cell line and freshly isolated bronchial epithelial cells. They found that infection of both cell types with *C. pneumoniae* increased IL-8 secretion in dose and time dependent manner (127). Another group, which has also endeavoured to elucidate the interactions between *C. pneumoniae* and airway epithelial cells, also demonstrated an increase in IL-8 expression by the A549 cell line (183). Further research also showed an increase in TNF-α and IFN-γ mRNA expression after infection with *C. pneumoniae*. However, it was only IL-8 protein that was detected in corresponding cell supernatants, whereas TNF-α and IFN-γ protein levels remained unaltered after infection (183). The expression of other cytokines important in inflammatory reactions such as IL-6 and IL-1β were also investigated but were undetected or remained unaltered between infected and uninfected cultures (183). However, in a continuous model of *C. pneumoniae* infection in HEp-2 cells, inflammatory cytokines such as IL-6 and IL-8 are significantly increased compared with uninfected cells (184).

Therefore, it is possible that the changes in cytokine expression by airway epithelial cells infected with *C. pneumoniae* may contribute to the pathogenesis of diseases such as asthma

and chronic obstructive pulmonary disease. Knowledge of the role of *C. pneumoniae* infection in the modulation of cytokine expression by airway epithelial cells is not comprehensive and warrants further investigation. Questions still remain unanswered regarding which cytokines are altered and what the mechanisms are that govern their modulation.

Phenotypic features of airway epithelium

The response of airway epithelial cells to *C. pneumoniae* infection is not restricted to modulation of cytokine expression. Cell surface molecules also play a role in inflammatory responses, promoting adherence between cells and activation and recruitment of inflammatory cells. Once infection has been established, in order to mount an efficient immune response T cell activation is required. For this to occur, three signals, provided through accessory cells are necessary; 1) adhesion between T cells and accessory cells, 2) antigen presentation, and 3) a secondary costimulatory signal. Professional and non-professional antigen presenting cells including, monocytes, macrophages and epithelial cells are capable of providing these signals via surface membrane expression of specific molecules (**Table 1.3**).

Adhesion molecule expression by airway epithelium

Adhesion between airway epithelial cells and T lymphocytes is mediated through intercellular adhesion molecule-1 (ICAM-1 or CD54) a molecule expressed on their surface (185). Leucocyte function-associated antigen-1 (LFA-1) is the counter receptor for ICAM-1 and is predominantly expressed on leucocyte subsets (186,187). In airway epithelial cells, ICAM-1 is upregulated by TNF-α, IL-1β, IL-4 and IFN-γ (155,185,188). The combination of IL-4 and TNF-α further increases ICAM-1 expression in bronchial epithelial cells (155). ICAM-1 expression on airway epithelial cells is also modulated by bacterial endotoxin and respiratory viruses (148,189,190).

Table 1.3: Cellular distribution and function of cell surface molecules.
LFA = lymphocyte function associated antigen; MHC = major histocompatibility complex; ICAM = intercellular adhesion molecule; IL = interleukin; NK = Natural Killer; HLA = human leucocyte antigen. Compiled from Barclay and colleagues (192).

SURFACE MOLECULE	CELLULAR DISTRIBUTION	FUNCTION
CD2 (LFA-2)	Lymphocytes	Adhesion Costimulation Enhances antigen recognition
CD3 (T cell receptor complex)	Lymphocytes	Antigen recognition Signal transduction Identifies T lymphocytes
CD4	T helper cells	Antigen recognition Identifies T helper lymphocytes
CD8	NK cells	Antigen recognition Identifies cytotoxic T lymphocytes
CD11a (LFA-1, α subunit of β_2 integrin)	Monocytes Neutrophils Lymphocytes	Adhesion
CD11b (Mac-1, α subunit of β_2 integrin, complement receptor type 3)	Neutrophils Monocytes	Adhesion
CD14	Monocytes Neutrophils Endothelial cells	Identifies cells of myelomonocytic lineage LPS receptor
CD16	Neutrophils	Distinguishes neutrophils from eosinophils
CD18 (βsubunit of integrin β_2)	Monocytes Neutrophils Lymphocytes	Adhesion
CD25 (α subunit of IL-2 receptor)	Monocytes Lymphocytes	Activation marker Activation & proliferation of T cells, NK cells, macrophages, B cells
CD28	Lymphocytes	Costimulation of T cells Signal transduction
CD40	Monocytes	Costimulation
CD54 (ICAM-1)	Monocytes Lymphocytes Neutrophils Airway epithelial cells Endothelial cells	Adhesion Costimulation
CD58 (LFA-3)	Monocytes Neutrophils Airway epithelial cells	Adhesion Costimulation Enhances antigen recognition
CD62L (L-selectin)	Neutrophils Monocytes Lymphocytes	Adhesion
CD69	Monocytes Lymphocytes Neutrophils	Activation marker
CD80 (B7-1)	Monocytes Airway epithelial cells	Costimulation of T cells Associated with Th1 responses
CD86 (B7-2)	Monocytes	Costimulation of T cells Associated with Th2 responses
MHC Class I (HLA-A, -B and -C)	Monocytes Lymphocytes Neutrophils Airway epithelial cells	Antigen presentation
MHC Class II (HLA-DR, -DQ and -DP)	Monocytes Activated lymphocytes Macrophages	Antigen presentation

An increased percentage of epithelial cells isolated from patients with asthma or chronic bronchitis, express ICAM-1 when compared to cells obtained from healthy control subjects (191). Furthermore, the expression of ICAM-1 was also shown to be correlated to the severity of disease. In a later study, the same group reported that that intensity of bronchial epithelial cell staining for ICAM-1 was increased from asthmatics compared to controls and was most likely due to an increase in the number of molecules being expressed by the cells (193). *Haemophilus influenzae* stimulation of bronchial epithelium also increases ICAM-1 (150). Soluble ICAM-1 expression by bronchial epithelial cells is also increased after exposure of the cells to cigarette smoke as compared to exposure to air (179).

Another molecule involved in cellular adhesion is CD58, also known as LFA-3. It is expressed widely in tissues including airway epithelium (194). CD58 is a member of the immunoglobulin family and mediates adhesion by binding to its counter receptor CD2 on T lymphocytes (195). There are two isoforms of CD58, one of which is a typical transmembrane receptor with a cytoplasmic tail and hydrophobic extracellular segment, and the second form is fixed within the cellular membrane, both of which interact with CD2 on leucocytes (195,196). Although CD58 is shown to be expressed on airway epithelial cell lines and primary bronchial epithelium, there is little extra information regarding the expression of this surface molecule in disease states (197,198).

Modulation of airway epithelial adhesion molecule expression by C. pneumoniae

Bronchial epithelial cells not only alter cytokine expression after *C. pneumoniae* infection, they also increase surface expression of adhesion molecules, such as ICAM-1 (127). Promotion of adherence between cells via ICAM-1 expression was corroborated by an increase in polymorphonuclear cell transepithalial migration after *C. pneumoniae* induced ICAM-1 expression (127). Further investigation suggested that the changes in cytokine (IL-8) and adhesion molecule expression (ICAM-1) were mediated by activation of the transcription factor NFκB as its activation was increased prior to induction of mRNA

expression both molecules (127). To date, there have been no studies investigating whether *C. pneumoniae* has any affect on the expression of CD58 by airway epithelial cells.

Antigen presentation by airway epithelium

Although airway epithelial cells are not professional antigen presenting cells they are involved in this process. After adhesion between potential antigen presenting cells and T lymphocytes, antigen presentation is the second step in T cell activation. Antigen presenting cells internally process antigens and present them as peptides. The peptides are then expressed on the cell surface coupled to major histocompatibility complex molecules (MHC). These molecules are divided into two main types - MHC class I and MHC class II. Major histocompatibility complex class I molecules in humans are also referred to as HLA-A, B or C and class II molecules also known as HLA-DR, HLA-DP or HLA-DQ (192). Major histocompatibility complex class I molecules are generally associated with presentation of peptides derived from endogenous sources such as those produced by viruses in infected cells or cellular proteins degraded within the cytoplasm and class II molecules present processed peptides from extracellular sources such as those derived from circulating proteins (195,199,200). Processed peptides are presented to T cells via MHC molecules and are associated with the T cell receptor complex (CD3) on the surface of T cells. Peptides coupled to MHC class I molecules are recognised by T cell receptors expressed on killer T cells (CD8 positive T lymphocytes), whereas peptides coupled to MHC class II molecules are recognised by T cell receptors expressed on helper T cells (CD4 positive T lymphocytes) (192,195,200).

Major histocompatibility complex class I molecules are expressed on practically all nucleated cells including respiratory epithelium, (190,201,202). The expression of MHC class I molecules on primary lung epithelial cells can be regulated by inflammatory mediators such as IFN-γ (190). Pathogens such as cytomegalovirus and rhinovirus, can also modulate MHC class I expression and infection of lung epithelial cells results in an increase in expression (190,201).

Bronchial, alveolar and small airway epithelial cells show high expression of MHC class II molecules (202,203). Other studies have shown that unstimulated primary lung epithelial cells express low levels of MHC class II molecules but this is significantly upregulated by incubation with IFN-γ (190,203).

Patients with asthma or chronic bronchitis show an increase in the percentages of cells expressing MHC class II molecules and there are predominantly ciliated bronchial epithelial cells (191). Furthermore, the intensity of staining in bronchial epithelial cells is increased compared to cells from normal patients and is localised to the apical surface of the cells (193).

Modulation of major histocompatibility molecule expression by airway epithelial cells after C. pneumoniae infection

The relationship between antigen presentation by airway epithelial cells infected with *C. pneumoniae* has thus far not been investigated. However, in a study investigating the effect of *C. trachomatis* infection on the expression of MHC Class I molecules, an airway epithelial cell line was employed (8). The airway epithelial cells showed a constitutive level of MHC Class I expression that was significantly inhibited after infection with *C. trachomatis*. MHC Class I expression could also be upregulated by IFN-γ in uninfected airway epithelial cells but the induced expression was decreased after infection with *C. trachomatis*. In an earlier study by the same group, utilising epithelial cells of mammary or cervical origin, infection of the cells with *C. trachomatis* had no effect MHC Class II expression (204). Interferon-γ significantly induced MHC Class II expression in uninfected cells but failed to do so in *C. trachomatis* infected cells. It was suggested that *C. trachomatis* inhibits MHC expression by degrading host cell transcription factors required for MHC expression (8,204). Failure to adequately express MHC molecules by chlmaydial infected cells may lead to diminished recognition and detection of the bacteria, leading to evasion of the immune system.

Costimulation molecule expression by airway epithelium

After presentation of antigen to T lymphocytes by antigen presenting cells, adequate activation of T lymphocytes requires a second costimulatory signal in addition to activation through the T cell receptor complex. If a secondary signal is not received, the T cell is not activated and enters into a state of anergy where it is unresponsive to additional antigenic stimulation (200,205).

CD80 and CD86 are costimulatory molecules, also referred to as B7-1 and B7-2 respectively. They are required for adequate activation of T lymphocytes and initiating an effective immune response. Both molecules bind to CD28 and CD152 (Cytotoxic T Lymphocyte Antigen-4; CTLA-4) on T cells and have a higher affinity for CD152 (192).

There are few papers providing information about CD80 and CD86 expression by airway epithelial cells and reports of expression are inconsistent. However, primary nasal and airway epithelial cells are shown by flow cytometry to express both molecules (206). Primary human alveolar epithelial cells and small airway epithelial cells have also been analysed by flow cytometry for CD80 and CD86 expression but failed to express either of the two costimulatory molecules (203). Primary bronchial epithelial cells are reported to express both CD80 and CD86 although the level of baseline expression of CD86 was only detected at low levels (201). This study also examined the effect of rhinovirus on these cells and demonstrated an increase in CD86 expression after infection, CD80 expression was not affected.

Treatment of primary nasal or airway epithelial cells with blocking antibodies to CD80 or CD86 leads to an inhibition of T cell proliferation, suggesting an active role of airway epithelial cells in interactions with T lymphocytes (206).

The airway epithelial cell lines, BEAS-2B and A549, have been shown to express both molecules by one group but other groups failed to confirm these results, as detected by flow cytometry (201,206,207). One group detected CD80 expression but not CD86 expression on A549 cells and the other did not observe expression of either costimulatory molecule on BEAS-2B cells (201,207). However, CD80 mRNA expression could be detected in BEAS-2B cells by northern blot analysis of total cellular RNA (207). Despite the uncertainty of baseline expression of CD86 on the A549 cell line, CD80 expression was shown to be modulated by rhinovirus and was significantly increased upon infection (201). Rhinovirus infection caused only minimal transient expression of CD86 by A549 cells.

Recently a new member of the B7 family, B7h, has been described in the mouse and its human equivalent, B7-H2 (208,209). Also, B7-H3 has also been discovered and is a recent addition to the human B7 family (210). Primary bronchial epithelial cells and BEAS-2B cells have been shown to express B7-H2 by flow cytometry (207). The surface expression of B7-H2 could be downregulated in both cell types by treatment with various cytokines including TNF-α, IL-4, and IFN-γ, although was not confirmed with further studies using western blot to examine protein expression. Polymerase chain reaction also detected gene expression for B7-H2 and B7-H3 by BEAS-2B cells.

CD40 is a costimulatory molecule that belongs to the TNF receptor superfamily and its counter receptor is CD154 (or CD40L), which is expressed on activated T lymphocytes (192). Primary bronchial epithelial cells obtained from normal human bronchi are reported to express low levels of CD40 by immunohistochemistry (211). The airway epithelial cell lines BEAS-2B and 9HTEo$^-$ are demonstrated to constitutively express CD40 (188,212). However, other studies show variable results with airway epithelial cell lines. A transformed fetal bronchial epithelial cell line, W126VA4 failed to stain positively with antibodies directed against CD40, whereas an adult bronchial lung carcinoma cell line, SKLU1, consistently

demonstrated positive results (211). CD40 expression on these cell lines and primary bronchial epithelium could be regulated by cytokines such as IFN-γ and TNF-α but this regulation was not observed in the 9HTEo$^-$ cell line (211,212). However, crosslinking of CD40 with a soluble form of its ligand, sCD40L induced expression of IL-8 and MCP-1 from 9HTEo$^-$ cells and increased surface expression of ICAM-1 (212).

Modulation of costimulatory molecule expression by airway epithelial cells after C. pneumoniae infection

To my knowledge there is no data examining the relationship between *C. pneumoniae* infection and airway epithelial costimulatory molecule expression.

Other responses of airway epithelial cells after infection with C. pneumoniae

Once adhesion and entry has been established the host cell may respond in a variety of ways. One of the defence mechanisms of the host cell is to undergo programmed cell death, or apoptosis. However, bacteria and viruses have evolved and adapted to this host cell response by altering the apoptotic pathway and possibly reducing the possibility of detection by the immune system. A number of papers demonstrate that *C. pneumoniae* is antiapoptotic. Apoptosis induced by staurosporine is demonstrated to be decreased in the HEp-2 cells and HL cells, also a human epithelial cell line, infected with *C. pneumoniae* (213,214). TNF-α induced apoptosis via the TNF receptor is also inhibited by HEp-2 cells infected with *C. pneumoniae* (213). Inhibition of staurosporine or TNF-α induced apoptosis by *C. pneumoniae* also inhibited cytochrome c release from mitochondria to the cytoplasm and caspase-3 activation was reduced. As release of mitochondrial cytochrome c is required for caspase activation, this data suggests that *C. pneumoniae* induces host cells to resist apoptosis upstream of cytochrome c secretion into the cytoplasm.

Inflammatory cells

Monocytes and alveolar macrophages

Physical properties of monocytes and alveolar macrophages

Monocytes play a key role in host defence mechanisms. They constitute 2-10% of circulating white blood cells and migrate into tissue at sites of inflammation where they differentiate into tissue macrophages. Monocytes have an eccentrically positioned nucleus and their cytoplasm contains a well developed Golgi apparatus and numerous mitochondria. One of their main functions is to phagocytose particulate matter in the form of cellular debris or invading micro-organisms in a non-specific manner (131).

Monocytes that have migrated to the lungs differentiate into alveolar macrophages. They are located in the interalveolar septum, form part of the alveolar surface at the luminal surface of the alveoli or are in the free alveolar space (131,132). Macrophages have an irregular shaped cytoplasm with protrusions which are important for movement and phagocytosis. Their cytoplasm contains few mitochondria and numerous secondary lysosomes due to their predominant function in phagocytosis and engulfment of potential pathogens and particulate matter that have reached the lower airways (131).

Susceptibility of monocytes and macrophages to C. pneumoniae infection

Cell lines of the monocyte/macrophage lineage including U937 and Mono Mac 6 cells are able to maintain the growth of *C. pneumoniae* (129,215). Although both cell lines support chlamydial growth, recovery of viable Chlamydia from U937 cells is less than that observed for the more commonly used HEp-2 epithelial cell line and long term cultures are not able to be established (129). Long term cultures are possible with the Mono Mac 6 cell line but the recovery of *C. pneumoniae* appears to decrease with extended culture and host cell viability decreases, thereby limiting further replication of *C. pneumoniae* (215).

It is well documented that freshly isolated human monocytes are susceptible to *C. pneumoniae* infection (129,216). It is however, unclear as to whether they can maintain

and support the growth of the bacterium. Freshly isolated monocytes from peripheral blood mononuclear cells are susceptible to infection with *C. pneumoniae* but the morphology of inclusions appear to be abnormal and significantly smaller than those found in parallel experiments using HL cells (human epithelial cell line) (216). A few mature normal EB's can be distinguished within the infected monocytes but RB's were clearly abnormal (216). Furthermore, progeny from infected cells are non-infectious and cannot be passaged into HL cells(216). Although it appeared that the monocytes in this study do not support the growth of *C. pneumoniae*, it was determined that the intracellular bacteria were still metabolically active.

There is little documentation regarding the growth and maintenance of *C. pneumoniae* in human macrophages. Peripheral blood mononuclear cell derived macrophages are susceptible to infection with *C. pneumoniae* and the growth sustained in the short term (129). The progeny from these cells were infectious but titres were significantly less that those obtained from parallel cultures of HEp-2 cells (129). Alveolar macrophages obtained from bronchoalveolar lavages samples are also shown to be susceptible to infection with *C. pneumoniae* (129,130). Macrophages obtained from COPD patients stain positively for *C. pneumoniae* by immunohistochemistry (217). Short term cultures show the presence of typical chlamydial inclusions but inclusions are significantly smaller in size and titres are less than those seen in comparison to corresponding HEp-2 cell cultures (129). Extended infection of alveolar macrophages with *C. pneumoniae* also results in a further decrease in observed inclusion number (130). Alveolar macrophages obtained from patients with confirmed *C. pneumoniae* pneumonia also demonstrate susceptibility and the presence of chlamydial inclusions (130).

Murine models of *C. pneumoniae* infection further demonstrate the susceptibility of macrophages to infection. Intranasal inoculation of mice with *C. pneumoniae* leads to the

detection of chlamydial DNA in alveolar macrophages, whereas intraperitoneal inoculation results in the detection of the bacteria in peritoneal macrophages (146,218). Murine alveolar macrophages are also able to support the growth of *C. pneumoniae* at the same level as parallel experiments in HEp-2 cells but infectious progeny was considerably less (129).

Cytokine expression by monocytes and macrophages

Monocytes are a rich source of a variety of cytokines. Interleukin-8 was originally isolated from the supernatant of lipopolysaccharide stimulated monocytes as a product with chemotactic activity for neutrophils and therefore designated monocyte derived neutrophil chemotactic factor (219). A number of other cytokines are also expressed by monocytes including IL-1α and IL-1β, IL-6, TNF-α, IL-10, IL-12 and GM-CSF (220-223) (**Table 1.2**). Monocytes can also be stimulated by LPS and IFN-γ to increase IL-1α, IL-1β, IL-6, IL-8 and IL-10 (220,221,223). Monocytes are responsive to the proinflammatory cytokines TNF-α and IL-1β and subsequently increase IL-8 (224). In contrast, the classically inhibitory cytokine IL-10 significantly decreases the expression of various monocyte cytokines including IL-1β, TNF-α, IL-6, IL-8 and IL-12 (223,225,226). IL-10 expression by monocytes is also reported to be decreased by the Th1 cytokine IFN-γ (227).

Alveolar macrophages are a major source of cytokines within the lungs and have the capacity to secrete a variety of cytokines including TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12 (228,229) (**Table 1.2**). Alveolar macrophages also increase production of cytokines in response to a number of different stimuli. Infection of alveolar macrophages with Respiratory Syncytial Virus results in increased expression of TNF-α, IL-6 and IL-8 (230). Similar results are obtained when cells are stimulated with LPS (228,230). LPS also increases the production of IL-1β, GM-CSF, IL-10 and IL-12 (222,228,229,231). Allergens such as swine dust, rye grass pollen and house dust mite also activate alveolar macrophages and increase production of IL-8, IL-1β and IL-6 (232,233).

Alveolar macrophages also respond to cytokines such as IL-10 and decrease their expression of TNF-α, IL-1β, IL-6 and IL-8 (228,234). IL-12 is inhibited by both IL-10 and TNF-α via separate pathways (235).

Macrophages are the predominant cell type in bronchoalveolar lavage fluid obtained from the airways of asthmatic patients (137,236). Although there is no difference in the number of macrophages in bronchoalveolar lavage fluid from normal and asthmatic subjects, the number of CD14 (a cell marker predominantly expression by both monocytes and macrophages) positive cells is increased and may be due to increased percentage of monocytes detected in bronchoalveolar lavage fluid from asthmatics (137,237). The presence of monocytes or macrophages within the airways may contribute to local inflammation by secretion of cytokines. The levels of a number of cytokines are increased in bronchoalveolar lavage fluid obtained from asthmatic airways and include IL-1β, IL-6, IL-8 and GM-CSF (137,237). Bronchoalveolar lavage fluid from community acquired pneumonia patients also show increased levels of IL-6 and IL-8 (238). Furthermore, the expression of these cytokines has been localised to monocytes or shown to be increased in asthmatics compared to healthy subjects (137,237). Alveolar macrophages from asthmatics show a similar cytokine profile with increased baseline levels of IL-6, IL-1β, and GM-CSF in comparison to alveolar macrophages from control subjects (137,233). After stimulation with LPS, alveolar macrophages from asthmatic patients secrete significantly more IL-8, GM-CSF and TNF-α in comparison to cells from healthy patients (222). Alveolar macrophages from community acquired pneumonia patients also exhibit increased mRNA expression for IL-8 compared to control patients (238). In addition, monocytes derived from peripheral blood of asthmatics also show increased expression of GM-CSF in comparison to normal patients at baseline and both GM-CSF and IL-1β after LPS stimulation(222). Peripheral blood monocytes from COPD patients express lower baseline levels of IL-8 compared to control subjects (239).

Upon stimulation with LPS levels of MCP-1 and IL-6 release are greater than that detected from monocytes from control patients (239). LPS stimulation of peripheral blood monocytes from COPD patients also show increased TNF-α and TGF-β expression when compared to control subjects (240).

The reported levels of IL-10 and IL-12 expression from asthmatic patients is conflicting. There is some evidence of decreased levels of IL-12 expression by whole blood monocytes from asthmatics compared to normal patients after stimulation with LPS (241). Similar results are observed after stimulation of peripheral blood with *Staphylococcus aureus* with decreased expression of IL-12 from cells of asthmatic patients (242,243). There is evidence that IL-10 production by stimulated monocytes from asthmatics is decreased compared with normal patients (241). These findings however are not consistent with other studies not showing any significant differences between asthmatics and normals (242). Levels of IL-10 measured in induced sputum from asthmatics and COPD patients are also reported to be decreased compared to healthy control patients (244). Cells exhibiting positive staining for IL-10 expression in this study appeared to be macrophages. Whereas, another study measuring IL-10 in BAL fluid, detected increased levels of cytokine IL-10 in bronchoalveolar lavage fluid from asthmatics (231).

Similar to studies in peripheral blood, the majority of studies investigating the levels IL-12 in the airways of asthmatics report that levels are decreased compared to those detected in the airways of normal patients. Specifically, IL-12 protein measured in bronchoalveolar lavage fluid recovered from asthmatic patients, is lower than that measured in fluid from normal subjects (245). These reduced levels are attributed to reduced expression by alveolar macrophages (233,245,246). However, other studies have shown an increased level of IL-12 protein in bronchoalveolar lavage fluid from asthmatics compared to normals (231).

Overall, the majority of papers report decreased expression of IL-12 and IL-10 in chronic airways disease. Since IL-12 stimulates IFN-γ production by T cells promoting Th1 responses. Thus, reduced IL-12 in asthmatics may be a reflection of the Th2 type immune response pattern predominantly associated with inflammatory processes chracteristic of asthma. However, IL-10 is described as an anti-inflammatory cytokine inhibiting major functions of monocytes and macrophages, including cytokine production. It is suggested that a polymorphism in the IL-10 gene promoter may be responsible for reduced expression in chronic respiratory disease (159). Reduced IL-10 expression in chronic respiratory disease may lead to an imbalance between pro- and anti-inflammatory processes, exacerbating disease states.

Monocyte and macrophage cytokine response to C. pneumoniae infection

Due to the association of *C. pneumoniae* with atherosclerosis and the presence of *C. pneumoniae* in atheromatous plaques (247), various groups have investigated the response of cells involved in the progression of this disease to chlamydial infection. Some of these cells such as monocytes and macrophages also play a major role in lung defence mechanisms. It is well documented that *C. pneumoniae* stimulates peripheral blood mononuclear cells to increase expression of TNF-α, IL-1β, IL-6 and IL-8 (248-251). Similarly, IL-10 and IL-12 are also reported to be increased in peripheral blood mononuclear cells after infection with *C. pneumoniae* (248,249,252). Monocytic cell lines such as U937 cells are also reported to increase IL-6 and IL-10 expression after infection with *C. pneumoniae* (252). The mechanisms of cytokine induction by *C. pneumoniae* infection in mononuclear cells are not fully understood. However, some investigations have suggested that chlamydial endotoxin and CD14 (the receptor for LPS) is not involved in modulation of TNF-α, IL-1β or IL-10 expression (249,250). Similarly in murine peritoneal macrophages induction of TNF-α and IL-1β by *C. pneumoniae* does not involve LPS binding protein, which facilitates binding of LPS to its receptor (249). Other research supports this phenomena by demonstrating that

C. pneumoniae induction of TNF-α in murine peritoneal macrophages is mediated by a heat labile protein (LPS is heat stable) (253). Further research showed that chlamydial heat shock protein 60 increases TNF-α and matrix metalloproteinase-2 and 9 (253).

Although alveolar macrophages are susceptible to *C. pneumoniae* infection, the interaction between host and pathogen in terms of immunological or physical response is not well documented. The proinflammatory cytokines TNF-α and IL-1β are demonstrated to be increased after *C. pneumoniae* infection, along with the neutrophil chemotactic cytokine IL-8 in human alveolar macrophages (130). Unlike other studies investigating the mechanisms of cytokine induction by *C. pneumoniae* in peripheral blood mononuclear cells (249,250), TNF-α secretion by alveolar macrophages was determined to be dependent on chlamydial endotoxin (130). Studies which have used monocyte derived macrophages show an increase in IL-6 production after stimulation with recombinant *C. trachomatis* HSP-60 and antigenic stimulation with *C. pneumoniae* also induced IL-6 production suggested to be via chlamydial HSP-60 (254).

Phenotypic features of monocytes and alveolar macrophages

Adhesion molecule expression by monocytes and alveolar macrophages

A variety of adhesion molecules are expressed by cells of monocytic origin including ICAM-1, CD11a and CD58 (187,194,255). Adhesion between monocytes or macrophages and T cells is required for initiating adequate immune system responses. Monocytes and macrophages in particular, are professional antigen presenting cells and require adhesion to T cells in order for this to happen. Cell surface molecules and their associated ligands are depicted in **Table 1.4**. As mentioned earlier, ICAM-1 interacts with CD11a which is mainly localised to leucocyte subsets including monocytes and macrophages (186,187). CD11a combines with CD18 to form leucocyte function-associated antigen-1 (LFA-1) and is a member of the β2 integrin subfamily (195). LFA-1 ligands also include ICAM-2 and ICAM-3. Monocytes express detectable levels of ICAM-1 when unstimulated but can be

SURFACE MOLECULE	LIGAND	FUNCTION
CD2 (LFA-2)	CD58 (LFA-3)	Adhesion Costimulation Enhances antigen recognition
CD3 (T cell receptor complex)	MHC molecules	Antigen recognition Signal transduction Identifies T lymphocytes
CD4	MHC Class II	Antigen recognition Identifies T helper lymphocytes
CD8	MHC Class I	Antigen recognition Identifies cytotoxic T lymphocytes
CD11a (LFA-1, α subunit of β_2 integrin)	ICAM-1 ICAM-2 ICAM-3	Adhesion
CD11b (Mac-1, α subunit of β_2 integrin, complement receptor type 3)	ICAM-1 Bacterial LPS Complement fragment iC3b	Adhesion
CD14	LPS LPS binding protein	Identifies cells of myelomonocytic lineage
CD18 (integrin β_2 subunit)	ICAM-1 ICAM-2 ICAM-3	Adhesion
CD25 (α of IL-2 receptor)	IL-2	Activation marker Activation & proliferation of T cells, NK cells, macrophages, B cells
CD28	B7 family (CD80, CD86)	Costimulation of T cells Signal transduction
CD40	CD154 (CD40 ligand)	Costimulation
CD54 (ICAM-1)	LFA-1 Rhinovirus receptor	Adhesion Costimulation
CD58 (LFA-3)	CD2	Adhesion Costimulation Enhances antigen recognition
CD62L (L-selectin)	Carbohydrates such as heparan sulphate	Adhesion
CD69	Unknown	Activation marker
CD80 (B7-1)	CD28 CD152 (CTLA-4)	Costimulation of T cells Associated with Th1 responses
CD86 (B7-2)	CD28 CD152 (CTLA-4)	Costimulation of T cells Associated with Th2 responses
MHC Class I (HLA-A, -B and -C)	T cell receptor complex on $CD8^+$ T cells	Antigen presentation
MHC Class II (HLA-DR, -DQ and –DP)	T cell receptor complex on $CD4^+$ T cells	Antigen presentation

Table 1.4: Ligands and major function of cell surface molecules.
LFA = ; MHC = major histocompatibility complex; ICAM = intercellular adhesion molecule; IL = interleukin; NK = Natural Killer; CTLA = cytotoxic T lymphocyte antigen; HLA = human leucocyte antigen; Compiled from Barclay and colleagues (192).

significantly increased after stimulation with PMA or IFN-γ (255,256). Monocytes obtained from COPD patients are less responsive to LPS compared to those collected from healthy controls (239). CD58 is widely expressed on cells including peripheral blood monocytes and alveolar macrophages (194,257). It is also known as LFA-3 and interacts with CD2 which is localised to T lymphocytes (258). Peripheral blood mononuclear cells can be induced to express CD58 by stimuli such as PHA (257). In response to this stimulus, cells expressing CD58 also increase their production of IFN-γ (257). Adhesion between antigen presenting cells and T lymphocytes via CD58 and CD2 is also suggested to increase antigen recognition by decreasing the distance between interacting cells (259).

Although CD40 is classified as a costimulatory molecule, its expression on activated monocytes is shown to mediate adhesion between these cells and cells expressing it's ligand, CD40L, such as activated T lymphocytes (260). CD40 is expressed on monocytes and its gene expression is increased by cytokines such as IFN-γ and GM-CSF (260). Stimulation of peripheral blood monocytes via CD40 activation results in increased expression of ICAM-1, antigen presentation and costimulation molecules and CD40 itself (261). Ligation of CD40 also stimulates monocyte cytokine release and may suggest that this surface molecule plays an important role in regulation of monocyte activity (261).

Modulation of adhesion molecule expression on monocytes and macrophages after C. pneumoniae infection

Adhesion molecule expression is also altered in U937 cells with an increase in ICAM-1 expression with increased infection with *C. pneumoniae* (252). The increase in ICAM-1 expression after *C. pneumoniae* infection of THP-1 cells (monocytic origin) was also shown to be associated with the differentiation of these cells into macrophages (255). However, in contrast to studies in monocytic cells, expression of adhesion molecules, ICAM-1, on the surface of alveolar macrophages is not altered by *C. pneumoniae* (130,252).

Antigen presentation by monocytes and alveolar macrophages

The second step involved in T lymphocyte activation involves presentation of processed antigen to the T cell by antigen presenting cells. Major histocompatibility complex class II molecules are expressed on normal peripheral blood monocytes and is shown to be increased by incubation with IL-4 or IFN-γ (262-264). Baseline expression of MHC class II and that induced by IL-4 and IFN-γ on monocytes is down regulated by the presence of IL-10 (264). Lipopolysaccharide stimulation of monocytes results in a dose dependent decrease in MHC class II expression and shown to be due to LPS induced IL-10 secretion from monocytes (223). Alveolar macrophages also show high expression of MHC class II molecules (265,266).

Modulation of MHC molecule expression by monocytes and macrophages after C. pneumoniae infection

Further, monocytic cells also respond to *C. pneumoniae* infection by modulating surface molecule expression. Infection of U937 cells with *C. pneumoniae* results in a decrease in major histocompatibility complex class I molecules which is suggested to be due to induction of IL-10 expression by these cells (252). Conversely, major histocompatibility complex class II molecule expression by alveolar macrophages is reported to be increased after infection with *C. pneumoniae* (130).

Costimulation molecule expression by monocytes and alveolar macrophages

In order for adequate T cell activation and an effective immune response, T cells also require a second costimulatory signal. The main pathway by which this occurs is through CD80 CD86 expression on antigen presenting cells and ligation with CD28 on T cells. CD80 is expressed at low levels on peripheral blood monocytes and alveolar macrophages from normal subjects (206,266). Activated T cells, alveolar macrophages, peripheral blood monocytes increase their expression of CD80 (267-269). The level of expression of CD80 on alveolar macrophages from asthmatic patients is unclear with some reporting increased expression of CD80 compared to control subjects, whilst others document a significantly

lower level of expression (246,270,271). In peripheral blood monocytes CD80 expression is reported to be lower in asthmatics compared to control patients (272). Stimulation of these cells with rhinovirus increased CD80 surface expression on monocytes but not to the same extent as that seen with cells from normal patients.

CD86 has a similar tissue distribution to CD80 but in contrast to CD80 is expressed at high levels on resting peripheral blood monocytes (206,267). IFN-γ stimulation of monocytes increases CD86 expression (273). In contrast to CD80, rhinovirus reduces CD86 expression on peripheral blood monocytes (272).

CD86 expression on alveolar macrophages and peripheral blood monocytes is similar between asthmatics and normal patients (270-272). However in sarcoidosis patients CD86 expression by alveolar macrophages is reported to be significantly higher in comparison to those obtained from normal patients (266).

An association between CD80 and CD86 expression and Th1 and Th2 immune responses respectively, has been suggested (274,275). In murine systems utilising blocking antibodies to CD80 or CD86 the cytokine profile of T helper cells is altered. Inhibiting the action of CD80 results in an increase in IL-4 production compared to cells treated with anti-CD86 antibodies or control cultures suggesting the involvement of CD86 is Th2 cytokine production (276). However, addition of anti-CD86 antibodies to cells causes an increase in IFN-γ suggesting a role of CD80 in the production of Th1 cytokines (276). However, in human systems, the role of CD80 and CD86 in Th1 and Th2 responses remains controversial. In Th1 and Th2 antigen driven systems, the role of CD86 in cytokine production and proliferation of peripheral blood mononuclear cells predominates (274). Blocking antibodies to CD80 had little effect either of these responses whereas inhibiting CD86 significantly downregulated the responses. The response of T cell clones to CD80 or CD86 blocking antibodies is not

affected, in terms of proliferation or cytokine secretion and suggested that there is no relationship between CD80 and CD86 and Th1 and Th2 responses respectively (274). In asthmatic patients, alveolar macrophages incubated with blocking antibodies to CD80 or CD86 caused similar inhibition of IL-4 expression from T cells suggesting a role for both molecules in Th2 cytokine production (271). Other studies support the hypothesis that CD80 and CD86 are associated with Th1 and Th2 immune responses. In allergic asthmatics the expression of CD86 is increased on B cells compared to nonatopic control subjects, whereas CD80 expression was similar between patient groups (277). Th2 cytokines such as IL-4 and IL-13 are also shown to modulate CD86 expression and cause an increase in expression on B cells but have no effect on CD80 expression (277). The Th1 cytokines IFN-γ and IL-12 had no effect on the expression of either costimulatory molecule and failed to inhibit CD86 expression increased by IL-4 (277).

One of the newest members of the human B7 costimulatory family, B7-H2 is also shown to be expressed on resting peripheral blood mononuclear cells by flow cytometry and western blotting (207). At present, no additional information is available about the expression of B7-H2 on alveolar macrophages or B7-H3 by monocytes or alveolar macrophages.

CD40 is part of the TNF receptor superfamily and is upregulated on monocytes by GM-CSF, IL-3 and IFN-γ (260). The interaction between CD40 and its ligand, CD40L on monocytes and T cells respectively also alters cytokine production and results in increased IL-12 secretion (278). Reduced IL-12 expression by alveolar macrophages from asthmatics correlates to reduced CD40 expression (246). Blocking antibodies to CD40 also inhibited IFN-γ along with IL-12 but increased IL-5 in co-cultures of alveolar macrophages and T lymphocytes suggesting an important role for CD40 in the regulation of Th1 and Th2 responses (246).

CD58 and CD2 are also suggested to be involved in costimulation of T cells. Although the CD58/CD2 pathway alone is not sufficient to activate T cells, the interaction does increase T cell activation by synergising with signals from the T cell receptor complex (195). T cells activated via the CD2 molecule together with ligation of the CD28 molecule, increase their production of IL-1α and TNF-α compared to crosslinking of either molecule alone (279).

The interaction between ICAM-1 and LFA-1 may also act as a costimulatory signal and results in increased activation of T cells via the T cell receptor complex (280). Costimulation mediated through ICAM-1 increases T cell proliferation as does LFA-1 but not to the same extent (281). It has also been suggested that ICAM-1 and LFA-1 interactions play a role in modulation of cytokine production by T cells. In a murine model, blocking antibodies to ICAM-1 in combination with ICAM-2 (which also interacts with LFA-1) caused an increase in Th2 cytokines IL-4 and IL-5 suggesting that these molecules may play a role in regulation of Th1 and Th2 development (282).

Modulation of costimulatory molecule expression by monocytes and macrophages after C. pneumoniae infection

There is no documentation available investigating the modulation of costimulatory molecule expression on monocytes or macrophages by *C. pneumoniae*. Expression of these molecules by antigen presenting cells facilitates effective activation of T cells and the role in which *C. pneumoniae* plays in this process requires investigation. Since the expression of CD80 and CD86 are suggested to be linked to Th1 and Th2 immune response patterns respectively, these studies may provide an indication as to whether *C. pneumoniae* infection leads to an imbalance between Th1 and Th2 immune responses. In addition, the ability of *C. pneumoniae* to modulate costimulatory molecule expression may be a potential point at which the immune system is compromised leading to inability to clear infection and possibly the development of persistence.

Other responses of monocytes and macrophages attributed to C. pneumoniae infection

C. pneumoniae infection of monocytes also alters cellular process such as programmed cell death. TNF-α induction of apoptosis is shown to be reduced in *C. pneumoniae* infected U937 cells (214). Similarly, peripheral blood mononuclear cells infected with *C. pneumoniae* show resistance to apoptosis induced by chemotherapy agents (248). Further research suggested that this was a result of *C. pneumoniae* induced IL-10 expression. However, contrary to this work, *C. pneumoniae* is also shown to be pro-apoptotic in other monocytic cell lines. THP-1 cells infected with *C. pneumoniae* show a rapid externalisation of phosphatidylserine (283). Phosphatidylserine is a lipid molecule localised to the plasma membrane of eucaryotic cells. It is usually found on the cytosolic side of the membrane but when the cell undergoes apoptosis it flips to the outside and serves as an early indication of cells undergoing apoptosis.

Lymphocytes

Physical properties of lymphocytes

Lymphocytes constitute approximately 20-30% of cells in circulating blood and can be split into two main cell types, B lymphocytes or T lymphocytes (132). B lymphocytes are predominantly involved in the humoral immune response and generation of immunoglobulins, whereas T lymphocytes are associated with cell mediated immunity. Approximately 80% of lymphocytes are in fact T lymphocytes (132) and are distinguished from B lymphocytes based on the expression of the cell surface marker CD3, which forms part of the T cell receptor (192). T lymphocytes can also be divided into T helper (Th) cells and cytotoxic T cells. These cells express cell surface markers CD4 or CD8 respectively. T helper cells can also be subdivided into Th1 or Th2 cells, characterised based on their cytokine expression profile (discussed later).

Susceptibility of T lymphocytes to C. pneumoniae infection

Currently, there are few publications documenting the presence of *C. pneumoniae* in T lymphocytes. *C. pneumoniae* DNA can however, be detected in $CD3^+$ T lymphocytes obtained from healthy blood donors or patients with coronary artery disease by nested PCR

(284). Immunohistochemistry and *in situ* hybridisation have also been used to detect *C. pneumoniae* in T lymphocytes in atherosclerotic plaques from the carotid arteries of patients diagnosed with atherosclerosis (16). Recently, peripheral blood lymphocytes were shown to not only to be susceptible to *C. pneumoniae* infection, but to also support the growth of this bacterium as evidenced by increased bacterial titres with extended culture periods (285). There are however, no publications addressing *C. pneumoniae* infection of lymphocytes in comparison to host cells more commonly associated with *C. pneumoniae* infection such as epithelial cells (HEp-2 cell line) (143,145). To our knowledge, there are no reports examining the presence of *C. pneumoniae* in lymphocytes from clinical samples of respiratory origin such as bronchoalveolar lavage or biopsy specimens.

Cytokine expression by T lymphocytes

Lymphocytes are a source of a wide range of cytokines. They can be subdivided into Th1 and Th2 cells based on their cytokine expression profile. Originally, Th1 and Th2 lymphocyte subsets were defined based on murine studies showing a distinction between two types of cytokine expression patterns by T helper cells (286,287). It has since been recognised that Th1 and Th2 subsets within human T lymphocytes is not so definitive. Th1 cells are characterised by their preference for production of IFN-γ, TNF-α and IL-2 (286). These cytokines drive cell mediated immunity and increase the cytotoxic activity of phagocytes upon exposure to pathogens such as bacteria and viruses (288,289). Whereas, Th2 cells primarily secrete IL-4, IL-5 and IL-10 (287). Th2 cytokines promote allergic inflammation, eosinophil activation and chemotaxis and further differentiation and development of Th2 type cells (287,290,291). A third subset, designated Th0 are reported to express a combination of both Th1 and Th2 cytokines (287). IL-12 is also classified as a Th1 cytokine produced by antigen presenting cells and acts on lymphocytes to promote the development of Th1 type cells (292,293). T cell lines cultured in the presence of IL-12 increase their production of IFN-γ whilst inhibiting IL-4 secretion (294).

Th1 cytokines such as IFN-γ are inhibitors of Th2 development and cytokine production. IFN-γ reduces IL-4 expression by B cells and IL-10 production by monocytes (160,161,227). Antigen induced eosinophil recruitment and activation in mouse models is inhibited by treatment with IFN-γ (295). Similarly, the actions of Th2 cytokines are inhibitory for Th1 immune responses. IL-10 has profound inhibitory effects of monocyte cytokine production and decreases IL-12 production which in turn leads to reduced lymphocyte IFN-γ expression (226). IL-4 also inhibits macrophage synthesis of IL-12 and IFN-γ (159,161).

The inflammatory component of asthma is characterised as a Th2 type response with increased IL-4 and IL-5 expression by T lymphocytes (166,296-298). In addition, IL-5 expression is correlated to increased eosinophil counts in bronchoalveolar lavage samples from asthmatics as compared to control subjects (297). A corresponding decrease in Th1 type cytokines is also observed. IFN-γ expression is reduced in asthma and correlated to disease severity (297,299,300). In addition, monocytes from atopic asthmatic patients show reduced monocyte and macrophage expression of IL-12 and decreased IL-12 dependent IFN-γ production by T lymphocytes (242,245).

In contrast to asthma, there is some suggestion that COPD patients exhibit an inflammatory pattern representative of a Th1 type response. Peripheral blood T cells from COPD patients show increased IFN-γ expression with a corresponding decrease in IL-4 expression in comparison to healthy controls (301). However, further research is required to confirm these findings. Nevertheless, lymphocyte cytokine expression is modulated in COPD and show increased levels of TNF-α and IL-1β in sputum and bronchoalveolar lavage samples (175,302). As mentioned above, these cytokines may be produced by T lymphocytes but monocytes and macrophages are often identified as the cell source of these cytokines in COPD patients (239). Further, COPD is associated with an intense neutrophil based

inflammatory response and therefore show increased levels of IL-8 production by monocytes, macrophages and airway epithelial cells (175,176,239,303).

Lymphocyte cytokine response to C. pneumoniae infection

The pattern of cytokine expression by T lymphocytes in *C. pneumoniae* infection may play an important role in determining the final outcome of infection. Typically, the clearance of infection is dependent on Th1 cytokine response patterns which is illustrated in patients with trachoma. In ocular infection due to *C. trachomatis*, patients who recovered from infection demonstrated increased Th1 type responses in comparison to patients who showed evidence of persistent chlamydial infection (304). In addition, peripheral blood mononuclear cells from patients with severe trachomatous scarring due to *C. trachomatis* infection, show reduced expression of Th1 cytokines (IFN-γ) and increased Th2 cytokine expression (IL-4) (305). Similarly in female patients with a history of pelvic inflammatory disease and a history of repeated infection with *C. trachomatis*, IFN-γ release into plasma is reduced after stimulation of peripheral blood with the chlamydial antigen HSP-60, compared to patients who had did not have a history of multiple *C. trachomatis* infection (306). However, in *C. pneumoniae* infection, the cytokine response pattern of T lymphocytes has not been thoroughly examined. Unstimulated peripheral blood lymphocytes collected from patients with acute respiratory tract infection secrete significant amounts of TNF-α and the Th2 cytokine IL-10 (307). However, upon stimulation with *C. pneumoniae* EB's IFN-γ production was induced and TNF-α and IL-10 were increased further (307). The balance of these cytokines, particularly IFN-γ production is thought to determine whether infection is cleared or if persistence is promoted (308).

Phenotypic features of lymphocytes

Adhesion molecule expression by T lymphocytes

T lymphocytes express a variety of surface molecules which are shown in **Table 1.3**. Resting lymphocytes express low levels of ICAM-1 but this is upregulated by IL-2 and IFN-γ

(309,310). The affinity for which ICAM-1 has for its ligand LFA-1 is increased by signals generated from crosslinking of the T cell receptor (311,312). In both adults and children with asthma, the percentage of lymphocytes expressing ICAM-1 is increased compared to healthy controls (313,314). The ligand for ICAM-1, is LFA-1 which is selectively expressed on leucocytes, including lymphocytes (187,315,316). ICAM-1 expression on lymphocytes therefore mediates adhesion between leucocytes.

LFA-1 is a member of the β_2 integrin family and is composed of CD11a and CD18 (195). Binding of LFA-1 with its ligands facilitates adhesion between leucocytes and non-inflammatory cells such as epithelial and endothelial cells which express ICAM-1 (187,317). CD11a expression by T lymphocytes may be upregulated by stimuli such as PHA but most regulation is suggested to occur by changes in the affinity of LFA-1 for its ligands (315). This may occur after T cell activation via TCR cross linking which results in a transient increase in affinity, or phorbol ester (PMA) activation, causing a sustained increase in affinity (311,312). The short-lived nature of this increased avidity via TCR activation allows reversible adhesion during interactions between lymphocytes and antigen presenting cells after recognition of antigen and communication with T cells is complete (311).

CD2 is expressed on practically all T lymphocytes (192,318). It is a monomeric transmembrane protein involved in both adhesion and signal transduction processes. The ligand for CD2 is CD58 (LFA-3) which is expressed on monocytes, macrophages, neutrophils and epithelial cells aiding adhesion between these cells (187,197,319). Adhesion via CD2 and CD58 ligation increases the strength of the physical interaction between lymphocytes and antigen presenting cells, thought to enhance antigen recognition.

The expression of CD62L on lymphocytes facilitates homing to lymph nodes. It is expressed by lymphocytes but expression is lost upon activation due to proteolytic cleavage (192,320).

Although there is no significant difference in the expression of CD62L on lymphocytes from asthmatics and controls (314), there are some reports suggesting a link with Th1 and Th2 phenotypes. One study reports that lymphocytes expressing CD62L predominantly express Th2 cytokines, IL-4 and IL-5. Whilst CD62L negative lymphocytes have a Th1 phenotype expressing IFN-γ (321). However, a later study documented that Th1 cells, generated by incubation of murine lymphocytes in the presence of IFN-γ, IL-12 and anti-IL-4, maintained CD62L expression (322). The expression of CD62L on these cells was dependent on the presence of IL-12.

Modulation of adhesion molecule expression by T lymphocytes after infection with C. pneumoniae

Although it is reported that lymphocytes are susceptible to infection with *C. pneumoniae* (285) and are activated to secrete cytokines (307), there has been no further analysis of the response of these cells to infection. There is some indication that ICAM-1 expression may be modulated by *C. pneumoniae* infection, as its expression is increased by *C. pneumoniae* infected monocytes (252,255). Analysis of lymphocyte adhesion molecule expression after *C. pneumoniae* infection may provide an insight as to whether cellular communication facilitated by these molecules is compromised, thereby modulating the host cell immune response possibly leading to persistent infection.

Lymphocyte surface molecules involved in antigen recognition

Foreign antigens are presented to lymphocytes via MHC molecules expressed on the surface of antigen presenting cells. These processed antigens are recognised via T cell receptor (TCR, CD3) expression on lymphocytes by an adhesion dependent process (311,323,324). T helper cells expressing CD4 mediate adhesion with and recognise foreign antigens bound to, MHC Class II molecules (311,325). Whereas, lymphocytes expressing CD8 mediate adhesion with and recognise antigen bound to, MHC Class I molecules (311,326).

Recognition and activation of lymphocytes via the TCR complex generates a number of intracellular signalling pathways. Ligation of the TCR increases phosphorylation of proteins and activation of the phosphoinositol pathway (311). In addition, increased inositol phosphate generation via TCR ligation leads to increased mobilisation of Ca^{2+} from intracellular stores (311,327). Activation of these pathways induces CD69 and CD25 expression which are markers of cellular activation (327-329).

In patients with asthma there is an influx of lymphocytes detected in bronchial biopsies with increases in both CD4 and CD8 subsets, compared with healthy control subjects (135). In addition, the percentage of activated lymphocytes isolated from patients with chronic respiratory disease is also increased as indicated by CD25 and CD69 expression (135,237,314). However, other studies analysing lymphocyte subsets in sputum samples from asthmatics, only show increases in the CD4 subset without any increase in CD8 lymphocytes (313). There are also some studies which do not detect any changes at all between lymphocyte subsets in normals and asthma or COPD patients (301,330). However, a number of researchers have detected an increase in the ratio between CD8 and CD4 lymphocytes in COPD patients compared with control patients (331-333).

C. pneumoniae modulation of lymphocyte surface molecules involved in antigen recognition

There have been few studies investigating whether *C. pneumoniae* infection in chronic lung disease alters the expression of lymphocyte surface molecules involved in antigen presentation. One group has observed a decrease in the ratio between lymphocytes expressing CD4 versus those expressing CD8 in a case study of bronchitis caused by acute *C. pneumoniae* infection (334). Whether similar patterns are observed in chronic airways diseases such as COPD and the correlation with disease severity remains to be elucidated.

Costimulatory molecule expression by T lymphocytes

Costimulatory signals provided by antigen presenting cells are predominantly mediated via the B7/CD28 pathway. In addition to presenting antigen to the T cell receptor complex by antigen presenting cells such as monocytes and macrophages, costimulatory signals are provided by the expression of CD80 and CD86. These molecules are ligands for CD28 and CTLA-4 (CD152) which are expressed on the surface of T lymphocytes (192). CD28 is constitutively expressed on approximately 80% of circulating T cells (335). In contrast, CTLA-4 is expressed on a low percentage of activated T cells but has a higher much affinity for the B7 molecules compared to CD28 (336,337).

Ligation of CD28, in combination with T cell receptor activation generates intracellular signalling cascades via tyrosine phosphorylation and activates both calcium dependent and calcium independent pathways (336). These pathways eventually lead to increased production of IL-2, IL-3, TNF-α, GM-CSF and IFN-γ (338,339). It is thought that increased cytokine production via the CD28 costimulation pathway is mediated by stabilisation of cytokine mRNA (335). CD28 costimulation also induces T lymphocyte proliferation, in cells which have been activated by the T cell receptor complex (340). IL-12 shows synergism with the CD28 pathway and is dependent on IL-2 expression, inducing lymphocyte proliferation and production of IFN-γ, TNF-α and GM-CSF (341).

Approximately 75% of $CD4^+$ lymphocytes in bronchial biopsies from asthmatics are reported to express CD28 (342), but there has been no comparison with healthy controls or other chronic respiratory disease states. CTLA-4 is expressed on a much lower percentage of lymphocytes (4%) from asthmatics but it is increased after viral infection (272,342).

CD2 and CD58 are also suggested to be involved in costimulation of T cells. Although the CD58/CD2 pathway alone is not sufficient to activate T cells, the interaction does increase T cell activation by synergising with signals from the T cell receptor complex (195). Activation

of T lymphocytes also increases the affinity with which CD2 associates with CD58 (319). Intracellular signals generated by CD2 ligation include protein kinase C activation, inositol phosphate production and increased cyclic AMP (318,343). Lymphocytes incubated with anti-CD2 antibodies transiently increase expression of TNF-α but a combination of CD2 and CD28 antibodies results in heightened TNF-α expression and induction of IL-1α (279).

The interaction between ICAM-1 and LFA-1 is also suggested to act as a costimulatory signal and results in increased activation of T cells via the T cell receptor complex (280). Costimulation mediated through ICAM-1 increases T cell proliferation as does LFA-1 but not to the same extent (281). It has also been suggested that ICAM-1 and LFA-1 interactions play a role in modulation of cytokine production by T cells. In a murine model, blocking antibodies to ICAM-1 in combination with ICAM-2 (which also interacts with LFA-1) causes an increase in Th2 cytokines IL-4 and IL-5 suggesting that these molecules may play a role in regulation of Th1 and Th2 development (282).

Another lymphocyte costimulatory pathway is mediated via CD40L ligation. Resting lymphocytes do not express CD40L but this ligand is induced upon activation (192). It is the ligand for CD40, which is expressed on monocytes and is upregulated by GM-CSF, IL-3 and IFN-γ (260). The interaction between CD40L and its receptor CD40, on T cells and monocytes respectively, also alters cytokine production and results in increased IL-12 secretion by monocytes (278). A similar observation has been reported in alveolar macrophages from asthmatic patients whose expression of CD40 and a corresponding IL-12 expression is decreased in comparison to healthy controls (246). Blocking antibodies to CD40 also inhibit IFN-γ along with IL-12 but increase IL-5 in co-cultures of alveolar macrophages and T lymphocytes suggesting an important role for CD40 in the regulation of Th1 and Th2 responses (246).

Modulation of costimulatory molecule expression by T lymphocytes after infection with C. pneumoniae

There are currently no reports published specifically addressing *C. pneumoniae* modulation of lymphocyte surface molecules involved in costimulation. An effective immune response to clear infection requires costimulatory signals to be provided to lymphocytes for adequate activation. Therefore, it is important to investigate not only the signals provided by antigen presenting cells in response to *C. pneumoniae*, but also the receptors on lymphocytes receiving the signals.

Neutrophils

Physical properties of the airway epithelium

Neutrophils are the most abundant type of white blood cell, constituting 60-75% of circulating leucocytes (132). Their size ranges between 12 and 15μm in diameter and are described as polymorphonuclear leucocytes, as their nucleus is multilobed giving the appearance of having more than one nucleus. Their cytoplasm contains numerous granules and are therefore also referred to as polymorphonuclear granulocytes. Two main types of granules exist in the neutrophil cytoplasm: azurophilic or primary granules and specific or secondary granules (131). Azurophilic granules contain enzymes such as elastase, myeloperoxidase, lysozyme, β-galactosidase, collagenase and defensins (132). Specific granules are more abundant and contain collagenase, alkaline phosphatase and lysozyme. Neutrophils are professional phagocytes and play a major role in host defence mechanisms engulfing foreign particles including cellular debris, bacteria and particulate matter in a non-specific manner (131). Foreign bodies are engulfed by neutrophils and enclosed within a membrane bound vesicle termed a phagosome. The phagosome fuses with cytoplasmic granules allowing the contents of azurophilic and specific granules to be released into the phagosome to ultimately result in killing micro-organisms (131,132). The neutrophil cytoplasm is scarce in other organelles but is rich in glycogen, which is broken down via glycolysis to provide a source of energy to the cells.

Neutrophil activation by stimuli, including invading micro-organisms, results in the production and release of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which play an important role in the clearance of infection (344). This process is referred to as respiratory burst. Activated neutrophils generate reactive oxygen species which are delivered to vacuoles containing phagocytosed bacteria and results in the destruction of the pathogen (344). However, larger pathogens such as fungi, which are unable to be phagocytosed are destroyed via release of oxidants from neutrophils in close proximity to the micro-organism (344).

Neutrophil infiltration and biological activity, at sites of infection and inflammation must be closely regulated so as to avoid inflicting damage on surrounding tissue by continued release of toxic mediators and perpetuating inflammation. Circulating neutrophils have a short life span and undergo programmed cell death (345). At sites of inflammation, resolution of inflammatory infiltrates containing neutrophils occurs by apoptosis or phagocytosis by tissue macrophages thus reducing neutrophil mediated tissue damage (345).

As neutrophils are highly motile and are recruited to sites of injury and inflammation, they are often attracted to the lungs of patients with inflammatory airways diseases. As a consequence, the percentage of neutrophils in sputum from COPD patients is significantly higher than those found in asthmatics or healthy volunteers (175,176,346). Although asthma is typically associated with increased eosinophil cell counts, in comparison to control subjects, asthmatics also have increased numbers of neutrophils present in BAL fluid and sputum (137,347). The levels of myeloperoxidase, used as an indicator of neutrophil activation is also increased in asthma and COPD patients but more so in the latter (176,178).

Susceptibility of neutrophils to C. pneumoniae infection

Neutrophils are key cells in the protection of the lungs against potential pathogens and are recruited early in the infection process (146), but few studies have investigated the

relationship between neutrophils and *C. pneumoniae* infection. Phagocytosis of chlamydial EB's by neutrophils was first observed almost 20 years ago with the *C. trachomatis* and *C. psittaci* species (348). However, it was only recently reported that *C. pneumoniae* is also ingested by polymorphonuclear leucocytes and was shown that the bacteria are not only engulfed by the cell, but remain viable (349). In addition, analysis of chlamydial LPS content of infected neutrophils by ELISA demonstrates that *C. pneumoniae* not only survives within neutrophils but multiplies within these cells (349).

Cytokine expression by neutrophils

Although neutrophils are not commonly regarded as cytokine producing cells there is increasing evidence that they secrete a range of cytokines when activated. Neutrophils stimulated with LPS secrete significant amounts of the chemokines IL-8, MIP-1α, MIP-1β (350,351). These cytokines are produced in a dose dependent manner with increasing concentrations of LPS causing increased levels of chemokine production (350,351). Similar to monocyte production of these chemokines, IL-10 is shown to have an inhibitory effect on the expression and release of these mediators (350,351). IL-10 acts by modulating the degradation and stability of mRNA transcripts (351). Treatment of peripheral blood neutrophils with IL-4 also decreases LPS induced IL-8 expression but to a lesser extent that that seen with IL-10 treatment (351). Low levels of TNF-α and IL-1β protein can also be detected in neutrophil supernatant after stimulation of cells with LPS (351). There is some controversy as to whether neutrophils are a source of IL-6 with some researchers reporting induction after stimulation with GM-CSF and TNF-α, but others not detecting IL-6 at all (351,352). The conflicting data is thought to be due to differences in culturing techniques and contaminating monocytes in the preparations, as they are a known source of IL-6 (351).

Cytokines classically described as Th1 and Th2 cytokines are also produced by neutrophils. Incubation of neutrophils with IL-12 significantly increase IFN-γ production, which is

subsequently increased further with TNF-α or LPS stimulation (353,354). Intracellular stores of IL-4 are also detected in neutrophils and secretion is significantly increased after activation with calcium ionophore (355).

In chronic airways disease the levels of IL-8, TNF-α and IL-1β are observed to be expressed at increased levels (166,175,222,302). However, as neutrophils are not commonly referred to as cytokine producing cells, the source of these cytokines is seldom investigated with regard to neutrophils. The inflammatory component of COPD is characterised by an influx of neutrophils into the airways and IL-8 has been localised to neutrophils in lung tissue samples from COPD patients (168,175,346). The presence of increased IL-8 in BAL from chronic bronchitis, asthmatics and COPD patients is also correlated to neutrophil cell counts (175,181).

During infection, due to chemokine release, an influx of leucocytes to the site of infection is evident and neutrophils are the predominant cell type in the early stages of infection, followed by significant monocyte migration (146,356). Therefore, the ability of neutrophils to secrete cytokines may play an important role in the regulation of the initial immune response to infection and prevailing inflammation.

Neutrophil cytokine response to C. pneumoniae infection

Overnight incubation of neutrophils with *C. pneumoniae* results in a dose dependent increase in IL-8 production (349) which may act as an autocrine loop in attracting additional neutrophils to the site of infection. GM-CSF expression was also assessed in the same study but it was not induced by *C. pneumoniae* infection of neutrophils (349). Apart from this study, the response of neutrophils to *C. pneumoniae* infection in terms of cytokine production has not been investigated. However, in animal models of *C. pneumoniae* infection, there is a significant influx of neutrophils to the site of infection (146,357). Neutrophils may therefore

contribute to the regulation and coordination of cytokine production during *C. pneumoniae* infection.

Phenotypic features of neutrophils

Adhesion molecule expression by neutrophils

Neutrophils express a range of surface molecules (**Table 1.3**), most of which are associated with adhesion mechanisms. Neutrophil expression of adhesion molecules regulates a number of cell functions including phagocytosis, granule release, respiratory burst and chemotaxis (187,358-360).

In order for neutrophils to participate in host defence in response to tissue injury and infection, they must first traverse the vascular endothelium. One of the key surface molecules involved in this process is CD62L (L-selectin) which facilitates adherence to endothelial cells (361). Neutrophils constitutively express CD62L but is expressed at a lower level on older neutrophils (362). Under normal conditions of adhesion and rolling of neutrophils along vessel walls CD62L is shed from the neutrophil surface by proteolytic cleavage. However, CD62L is also downregulated on neutrophils by a number of inflammatory mediators including GM-CSF, TNF-α, LPS and leukotriene B4 (363,364).

Neutrophils obtained from blood or sputum of mild or severe asthmatics show similar levels of CD62L expression, compared with those obtained from control subjects (365). There is however, reduced CD62L expression on neutrophils obtained from sputum as compared to those from peripheral blood (365). This difference in expression between sputum and blood neutrophils is thought to be due to migration rather than inflammatory processes associated with airways disease. Neutrophils from BAL samples of asthmatics are also shown to express similar levels of CD62L before and after allergen challenge (366). Neutrophils are thought to be the main cell involved in the inflammatory process underlying COPD with increased recruitment and activation of these cells in the airways (175,176,346). However, the

expression of CD62L on neutrophils isolated from peripheral blood collected from COPD patients is not significantly different to that detected on neutrophils from healthy controls (367). In addition, during exacerbations of COPD, neutrophil expression of CD62L is not changed compared with stable COPD patients (367).

A second molecule involved in migration of neutrophils from the circulation is CD11b, which is the alpha chain of the β_2 integrin, CD11b/CD18 (187). It is also known as Mac-1 or complement receptor type 3 (CR3) (192). The ligand for CD11b/CD18 associated with adhesion processes is ICAM-1 but it also binds complement fragment iC3b, heparin and fibrinogen (192). Peripheral blood neutrophils constitutively express CD11b at a high level (363,368). Incubation of cells with GM-CSF, IFN-γ or TNF-α results in significant increases in neutrophil expression of CD11b (363,368,369). LPS is also demonstrated to increase CD11b expression via Toll-like receptor activation on neutrophils (364).

The expression of CD11b on resting neutrophils from patients with mild or severe asthma is similar to that seen on neutrophils from healthy controls (365,370). In contrast to CD62L expression, sputum neutrophils from asthmatics or controls exhibit a higher level of CD11b expression compared to their blood counterparts (365). Stimulated neutrophils from asthmatics significantly increase CD11b and the response is greater than that seen with neutrophils from healthy controls (370).

In contrast to asthma, baseline neutrophil expression of CD11b is increased in stable COPD patients compared to controls (367,371). In addition, CD11b and CD18 expression on neutrophils derived from sputum obtained from patients with airflow obstruction, are inversely correlated to lung function (FEV_1/FVC ratio) (371). However, during COPD exacerbations, CD11b/CD18 expression is reduced on peripheral blood neutrophils compared with stable patients (367).

Another member of the β_2 integrin family expressed by neutrophils is CD11a. As mentioned earlier, CD11a combines with CD18 to form LFA-1 (195). Although CD11a is expressed on peripheral blood neutrophils, its expression is largely unaffected by inflammatory mediators (361,372). As a consequence, the majority of research has focussed on neutrophil expression of CD11b. However, a few studies have addressed the question of CD11a expression by neutrophils in chronic airways diseases. Sputum neutrophils from smokers with airway obstruction, show similar levels of CD11a expression in comparison to patients without obstruction (371). Likewise, circulating neutrophils from COPD patients express LFA-1 at a level similar to that of matched controls (367). However, LFA-1 expression is decreased during exacerbations of COPD compared to stable patients (367).

LFA-1 positive neutrophils mediate cellular adhesion with cells that express ICAM-1 (CD54) (187). ICAM-1 is detected at low levels on unstimulated neutrophils, mediating homotypic cellular adhesion and neutrophil aggregation (310,373). Neutrophil expression of ICAM-1 may also facilitate adhesion between other leucocytes expressing LFA-1 such as lymphocytes promoting cellular communication between leucocyte subsets. The ability of neutrophils to become activated and participate in inflammatory processes is largely dependent on adhesion to and migration from the vasculature, which is predominantly mediated via β_2 integrins. Therefore, the majority of research into neutrophil adhesion molecule expression has focussed on CD11b/CD18 and as a consequence there is little information regarding the regulation of ICAM-1 expression on neutrophils. However, neutrophil expression of ICAM-1 is upregulated in response to inflammatory mediators such as TNF-α and GM-CSF (374). Further, pathogens such as *Staphylococcus aureus* are also shown to increase peripheral blood neutrophil ICAM-1 expression, suggested to be mediated via TNF-α production (374). The responsiveness of neutrophils to TNF-α may be due to NFκB and AP transcriptional regulation of ICAM-1 (375). Binding between LFA-1 and ICAM-1 on neutrophils may also

play a role in the activation of these cells. Crosslinking of ICAM-1 on neutrophils with antibodies increases CD11b expression with a corresponding decrease in CD62L expression (376). In addition, the same study showed that ICAM-1 crosslinking increased neutrophil oxidative burst as indicated by H_2O_2 formation (376).

Modulation of adhesion molecule expression on neutrophils after C. pneumoniae infection
Although there have been no previous studies published with regard to *C. pneumoniae* modulation of neutrophil surface molecule expression, the involvement of these molecules in major neutrophil functions may be a potential target for modulation. Numerous studies have shown that *C. pneumoniae* infection of cells other than neutrophils, particularly epithelium and endothelium cause the release of IL-8 and result in transepithelial or transendothelial migration of neutrophils to the site of infection (127,356,377). The role of adhesion molecule expression in this process is demonstrated by blocking *C. pneumoniae* induced ICAM-1 expression on epithelial cells, which significantly inhibits migration (127). This prompts the question as to whether neutrophil adhesion molecules are also involved in this process and if they are regulated by *C. pneumoniae* infection. Infection of endothelial cells with *C. pneumoniae* results in increased adhesion of neutrophils and monocytes to infected cells and is blocked by antibodies to β_2 integrins CD11a, CD11b and CD18 (141). In addition, in a murine model of *C. trachomatis* infection migration of neutrophils to the site of infection is shown to depend on the β_2 integrin subunit CD18 (378). These studies suggest the involvement of specific adhesion molecules in adherence to *C. pneumoniae* infected cells but the direct regulation of the expression of these molecules by *C. pneumoniae* has not been investigated.

Antigen presentation and costimulatory molecule expression by neutrophils
Resting neutrophils do not express MHC Class II molecules or express costimulatory molecules of the B7/CD28 pathway, such as CD80 and CD86. These antigens can be detected in intracellular stores of unstimulated neutrophils but are not detected on the cell

surface (379). Recent studies suggest however, that under the right conditions neutrophils may acquire this phenotype that is more commonly associated with professional antigen presenting cells such as monocytes and dendritic cells. Incubation of neutrophils with GM-CSF and IFN-γ induce MHC Class II, CD80 and CD86 expression (380,381).

Modulation of antigen presentation and costimulatory molecule expression by neutrophils after C. pneumoniae infection

As mentioned above, neutrophils are not commonly referred to as antigen presenting cells or provide costimulatory signals to lymphocytes. At present, there is no documentation available investigating the modulation of antigen presentation or costimulatory molecule expression on neutrophils by *C. pneumoniae*.

Other responses of neutrophils to C. pneumoniae infection

Neutrophils undergo spontaneous apoptosis *in vitro* (345). Similar to monocytes and macrophages, *C. pneumoniae* infection of neutrophils inhibits apoptosis in neutrophils and prolongs their survival (349). In comparison to uninfected neutrophils which undergo apoptosis within 10 hours, cells infected with *C. pneumoniae* are shown to survive for up to 90 hours (349). The mechanism by which *C. pneumoniae* interrupts neutrophil cell cycle is via reduced caspase activity which is known to be pro-apoptotic (349,382).

Summary

C. pneumoniae is a pathogen that predominantly infects humans and is a common cause of a wide range of acute and chronic diseases of the respiratory tract. It is a unique intracellular bacteria with a biphasic developmental cycle, alternating between infectious and non-infectious forms. *C. pneumoniae* may also develop into a persistent state with the formation of aberrant bodies and prolongs its association with the host cell. It is becoming more evident that chronic airways diseases such as asthma and COPD are associated with chronic or persistent *C. pneumoniae* infection. Despite this, chronic *C. pneumoniae* infection may go undetected as patients are often asymptomatic.

Chronic respiratory disease is associated with an underlying inflammation involving a variety of cells including the airway epithelium, neutrophils, lymphocytes, monocytes and macrophages. Changes in the airways of patients with chronic respiratory disease include damage to the respiratory epithelial lining, transmigration of leucocytes from the circulation and infiltration into the lung tissue and an imbalance of both pro- and anti-inflammatory mediator release from a variety of cells. Epithelial cells and leucocytes are a source of cytokines and chemokines which are involved in host defence mechanisms regulating chemotaxis, phagocytosis and cytotoxic activity of inflammatory cells. In order to maintain the integrity of the immune system involved in protecting the lungs from invading pathogens and noxious stimuli, these cells must be under strict regulation.

Since a variety of cells within the lungs are susceptible to *C. pneumoniae* infection, including the respiratory epithelium, alveolar macrophages, monocytes, lymphocytes and neutrophils, the response of these cells to infection may contribute to the underlying inflammation associated with chronic respiratory disease. The airway epithelium is the primary site of *C. pneumoniae* infection but the response of these cells to infection has not been comprehensively studied. This specialised lining of cells is a source of cytokines and express a variety of surface molecules which participate in initialising and orchestrating defence mechanisms to fight infection. The epithelium has the capacity to recruit leucocytes to the site of infection in an attempt to defend the lungs against invading pathogens and clear infection. However, the response of the airway epithelium to *C. pneumoniae* infection may be compromised and result in modulation of defence mechanisms allowing chronic *C. pneumoniae* infection to develop. Research within this field has focussed on the production of chemokines specific for neutrophil chemotaxis but a detailed study of other responses is required. These studies will enable us to answer questions about inflammatory mediators released and the changes in surface molecule expression which have an impact on

surrounding epithelial cells as well as other cells involved in inflammatory processes. The airway epithelium is in a prime position for *C. pneumoniae* to manipulate and orchestrate the response of inflammatory cells and may promote an environment that is favourable for the development of persistent infection.

Although there is information regarding the response of monocytes and macrophages to *C. pneumoniae* infection, most studies have utilised cell lines or a heterogenous cell population from peripheral blood. Therefore, identification and a thorough examination of whether cytokine expression profiles are modulated by *C. pneumoniae* infection in terms of pro- and anti- inflammatory mediators is also required. It has only been recently recognised that lymphocytes are susceptible to and support the growth of *C. pneumoniae*. Examination of the cytokine expression profiles of these cells in response to *C. pneumoniae*, particularly in terms of Th1 and Th2 type responses, may provide an insight into how persistent infection develops. It will be important to examine whether *C. pneumoniae* modulates cytokine expression by cells susceptible to infection and whether the patterns detected mimic those that are observed in chronic respiratory disease. This may provide knowledge and an understanding of the contribution of *C. pneumoniae* to the underlying inflammation associated with chronic lung diseases.

A second arm, in an attempt to understand how persistent *C. pneumoniae* infection may develop, is whether *C. pneumoniae* modulates the expression of host cell surface molecules. These molecules facilitate a number of cellular processes which enable communication between cell types and are essential to the immune system to efficiently fight and clear infection. Few previous studies have investigated this aspect of the interaction between *C. pneumoniae* and susceptible host cells. There are three key processes required for adequate T cell activation and ability to clear infection. These processes of adhesion, antigen presentation and costimulation are mediated by the expression of surface molecules on a

range of cells including airway epithelium, monocytes, neutrophils and lymphocytes. The level of surface marker expression may be modulated in response to cytokines, viruses and bacteria. We are therefore interested to examine whether these processes are modulated by *C. pneumoniae* infection by measuring the expression of surface molecules which facilitate these processes. Expression of cell surface molecules also provide an indication of cellular activation. The ability of *C. pneumoniae* to alter the activation status of inflammatory cells may also contribute to increased cellular activation of cells seen in chronic lung disease. Interruption or modulation of one or more of these processes may disrupt the ability of the immune system to detect and react to *C. pneumoniae*, eventually leading to persistent infection.

The studies documented herein have been undertaken to investigate the basic immune responses of cells susceptible to *C. pneumoniae* infection in terms of cytokine and surface marker expression profiles. A study of cytokine profiles will also provide information as to whether a predominance of Th1 versus Th2 patterns exist, particularly in monocytes and lymphocytes. An understanding of the relationship between host cell and *C. pneumoniae* in terms of cytokine expression may contribute to the underlying inflammation associated with chronic respiratory disease. Since the expression of surface molecules are an indication of cellular activation and mediate a number of cellular processes involved in the regulation of the immune response, we have also examined if *C. pneumoniae* modulates the expression of these molecules. These studies will provide an understanding of the basic immune response to acute *C. pneumoniae* infection and investigate possible mechanisms as to how persistent *C. pneumoniae* infection develops.

CHAPTER 2

METHODS

Airway epithelial cell lines and culture conditions

HEp-2 cells

HEp-2 cells, a tracheal carcinoma cell line (ATCC No CCL-23), were used for the propagation and culture of *C. pneumoniae*. The cells were maintained in Dulbecco's Modified Eagles Medium (Gibco BRL Life Technologies, Grand Island, NY, USA) with 20mM HEPES buffer (ICN Biomedicals, Irvine, CA, USA), supplemented with 10% foetal calf serum (FCS) (Trace Biosciences, Melbourne, VIC, Australia), 2mM L-glutamine, 100μg/ml streptomycin sulphate and 2μg/ml gentamicin (all purchased from Gibco BRL Life Technologies) (complete DMEM). Confluent cell monolayers were passaged by rinsing with 1 X Phosphate Buffered Saline (PBS) before incubation of cells with 0.1% trypsin at 37°C. Flasks were then rinsed with complete DMEM and cells centrifuged at 200 x *g* for 5 minutes. Supernatant was discarded and cell pellet resuspended in complete DMEM before returning to 75cm^2 tissue culture flasks and grown at 37°C in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. These cells were routinely passaged 1 in 6 for up to 30 passages.

16HBE14o- and 16HAEo- cells

16HBE14o- and 16HAEo- cell lines were kind gifts from Dr D Greunert (National Institute of Health Cystic Fibrosis Research Center, University of California, San Francisco) and were utilised for investigating the host cell immune response to *C. pneumoniae* in terms of cytokine production and surface marker expression. Both 16HBE14o- and 1HAEo- cell lines are derived from human bronchial epithelium and are transformed with SV40 large T-antigen (383,384). Cells were maintained in RPMI 1640 media (Gibco BRL Life Sciences) supplemented with 10% FCS, 100μg/ml streptomycin sulphate and 2μg/ml gentamicin, 2mM L-glutamine. Confluent cells were passaged by first rinsing with 1 X PBS followed by incubation with 0.1% trypsin at 37°C to disrupt cell monolayers. Flasks were then rinsed with

complete RPMI 1640 media before centrifugation at 200 x *g* for 5 minutes. Cells were then resuspended in complete RPMI 1640 media and returning to 75 cm^2 culture flasks and grown at 37°C in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. These cell lines were routinely passaged 1 in 2 for up to 30 passages.

BEAS-2B cells

BEAS-2B cells (American Type Culture Collection CRL-9069) were originally derived from bronchial epithelium and transformed with SV40 large T-antigen and were used to investigate the modulation of cytokine and surface marker expression in response to *C. pneumoniae*. The cell line was maintained in Keratinocyte Serum Free Media supplemented with Bovine Pituitary Extract and Epidermal Growth Factor according to manufacturers instructions (Gibco BRL Life Technologies) and 100μg/ml streptomycin sulphate, 2μg/ml gentamicin and 2mM L-glutamine. Confluent cells were passaged by first rinsing with 1 X PBS followed by incubation with 0.1% trypsin at 37°C to disrupt cell monolayers. Flasks were then rinsed with complete Keratinocyte-SFM before centrifugation at 200 x *g* for 5 minutes. Cells were then resuspended in complete Keratinocyte-SFM and returning to 75 cm^2 culture flasks and grown at 37°C in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. These cells were routinely passaged 1 in 2 for up to 30 passages.

Cryopreservation of cells

In order to maintain stocks of cell lines, confluent cells were frozen by cryopreservation and stored in liquid nitrogen storage vessels. Freezing medium for all cell lines except BEAS-2B cells consisted of a mixture of 50% (vol/vol) of the appropriate base medium (DMEM or RPMI) with 30% vol/vol FCS and 20% (vol/vol) dimethylsulphoxide, filter sterilised through a 0.22μM filter and stored at -20°C until required. Cells to be frozen were trypsinised by the standard protocol and resuspended in complete media at a concentration of $1x10^7$ cells/ml. An equal volume of freezing media was then slowly added (drop wise) to the cells with

continual swirling to ensure thorough mixing. Cells were then aliquotted into cryotubes and transferred to -20°C freezer for 1 hour. Cells were then transferred to -70°C for 1 hour before long term storage in liquid nitrogen.

Freezing medium for cryopreservation of BEAS-2B cells consisted of the normal growth media (K-SFM with supplements) containing 5% dimethylsulphoxide (vol/vol). Confluent flasks of BEAS-2B monolayers were trypsinised by the standard protocol and resuspended in freezing media with a final concentration of $3x10^6$ cells/ml. Cells were then aliquotted into cryotubes and transferred to -70°C overnight in a controlled rate freezing vessel (Nalgene Cryo 1°C freezing container, Nalge Nunc International, Rochester, NY). Cells were then transferred to liquid nitrogen for long term storage.

Revival of cryopreserved cells

All cells were revived by thawing vials in a 37°C water bath and transferred to a centrifuge tube containing the appropriate complete media. This was then slowly diluted 1:1 with complete medium, followed by centrifugation for 5 minutes at 200 x *g*. Pellets were then resuspended in 5ml complete medium and transferred to $25cm^2$ tissue culture flasks and incubated overnight at 37°C in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. The next day media was discarded and replaced with fresh complete media and cells cultured until confluent and passaged into $75cm^2$ tissue culture flasks.

Collection of whole blood

Whole blood was used to investigate the response of monocytes, neutrophils and T lymphocytes to *C. pneumoniae* stimulation in terms of cytokine production and surface molecule expression. Whole blood was collected from volunteers who were non atopic, non smokers with no history of respiratory disease. Venous blood was collected into tubes containing 20 units/ml sodium heparin as an anticoagulant (David Bull Laboratories,

Mulgrave, VIC, Australia). Blood was diluted 1:1 with serum free DMEM, supplemented with 100μg/ml streptomycin sulphate, 2μg/ml gentamicin and 2mM L-glutamine, before incubating overnight with desired treatment for stimulation of leucocytes.

Approval for collection of blood was granted by the Women's and Children's Hospital Ethics committee.

Detection of C. pneumoniae specific antibodies in serum

To assess the status of *C. pneumoniae* infection of subjects donating whole blood for the documented studies, serum was screened for the presence of *C. pneumoniae* specific antibodies. Serum was prepared by collecting 2ml of whole blood into a 4ml Vacuette® tube (containing Z serum separator and Clot Activator; Greiner Bio-One Incorporated, Longwood, FL) and allowing to clot at room temperature. Serum samples were then centrifuged at 1500 x *g* for 10 minutes at room temperature. Serum was then transferred to sterile 1.5ml microcentrifuge tubes and stored at -70°C until assayed.

Serum samples were assayed for the presence of IgG and IgM antibodies to *C. pneumoniae* using the micro-immunofluorescent antibody (MIF) test. This test was performed by experienced laboratory personnel in the Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, SA, Australia. The presence of serum antibodies were detected using Chlamydia MIF IgG and Chlamydia MIF IgM tests (Focus Technologies, Cypress, CA) run in parallel. The MIF test is an indirect immunofluorescent two-step 'sandwich' assay. The first step involves incubation of the serum samples with purified Chlamydial EB's on glass slides. Each slide consists of 12 wells, with each well containing four individual spots for the three chlamydial species (*C. pneumoniae*, *C. trachomatis* and *C. psittaci*) and a yolk sac control. After washing, the second step involves incubation of the slides with the appropriate fluorescent conjugate (anti-human IgG fluorescein or anti-human IgM fluorescein). Slides are washed and dried before observing under a fluorescence

microscope with a 470-490nm (absorption wavelength) filter (emission wavelength 520-560nm) at a final magnification of 400X.

Serum samples are diluted 1:16 in PBS or 1:10 in IgM Pretreatment Diluent for IgG and IgM determination respectively. Incubation of serum samples with IgM Pretreatment Diluent decreases competition between serum IgM and IgG antibody that may result in false negatives. The presence of rheumatoid factor (complexed IgG) in serum may result in false positives and therefore serum is incubated with IgM Pretreatment Diluent to remove IgG and complexed IgG. Undiluted Positive control is added to the first well and undiluted Negative control to the second well and diluted sera to subsequent wells of the IgM row only. Slides are then incubated for 60 minutes at 35-37°C. Undiluted controls and diluted sera samples are then added to appropriate wells of the IgG row and slides returned to incubate for a further 30 minutes at 35-37°C. Slides are then rinsed with PBS, one row at a time to avoid contamination of specimens. Slides are then washed in PBS with gentle agitation for 10 minutes. Slides are then briefly dipped into deionised water and gently blotted onto clean blotting paper. IgG Conjugate and IgM Conjugate are then added to all wells of the appropriate rows and incubated in a humidified atmosphere for 30 minutes at 35-37°C. Slides are washed as previously described and covered with a coverslip using the provided Mounting Media. Where possible the slides are read the same day, otherwise are stored at 2-8°C in the dark until the following day.

All tests were performed as single serum tests with previous or past infection with *C. pneumoniae* defined as an IgG serum specific antibody titre at a dilution of 1:16 and an indication of primary infection with the detection of IgM serum specific antibodies at a dilution of 1:10. Tests were considered valid if all positive controls exhibited moderate to high fluorescence with all antigen spots and negligible reactivity with the yolk sac control and the negative control showed no fluorescence with all spots.

C. pneumoniae strains, propagation and harvesting

Propagation

Two *C. pneumoniae* isolates were used in the studies documented herein. The first *C. pneumoniae* isolate IOL-207 was originally isolated from a patient with trachoma (385). This isolate has since been sequenced and is characterised as *C. pneumoniae* (27). The second *C. pneumoniae* isolate employed later in the current studies was an Australian isolate, WA97001, obtained from a nasopharyngeal aspirate (386). Both isolates were propagated in the HEp-2 cell line based on the methods described by Mathews and colleagues (68) (**Figure 2.1**). Cells were seeded at $1x10^6$ cells/ml in $75cm^2$ flasks in complete DMEM. The following day, medium was replaced with 5ml complete DMEM plus *C. pneumoniae* inoculum. Flasks were centrifuged at 640 x *g*, for 1 hour at 25°C (Beckman GS-6R) before returning to the incubator overnight. Inoculum was then replaced with 10ml complete medium plus 1μg/ml cycloheximide (Sigma, St Louis, MO, USA). Cycloheximide is routinely added to culture medium to induce host cell cytostasis which results in increased growth of *C. pneumoniae* (145). The infection period of HEp-2 cells inoculated with the WA97001 isolate was continued until 72 hours post inoculation. However, medium from HEp-2 cell cultures infected with the IOL-207 isolate was changed 72 hours post inoculation and grown for a further 3 days. At the end of the infection period an equal volume of sucrose phosphate glutamine (SPG) buffer (0.2M sucrose, 3.8mM KH_2PO_4, Na_2HPO_4, 5mM L-glutamic acid, pH 7.4) was then added to flasks before freezing at -70°C.

Harvesting

Flasks of infected HEp-2 cells, previously frozen at -70°C were thawed slightly under warm water before addition of sterile glass beads (**Figure 2.2**). Chlamydia was then harvested by mechanical disruption by vortexing the flasks for 5 minutes. Flasks were rinsed with 5ml SPG buffer and contents transferred to a centrifuge tube on ice. Cellular debris was pelleted by an initial centrifugation at 3000 x *g*, for 5 minutes at 4°C (Beckman, JA-14 rotor).

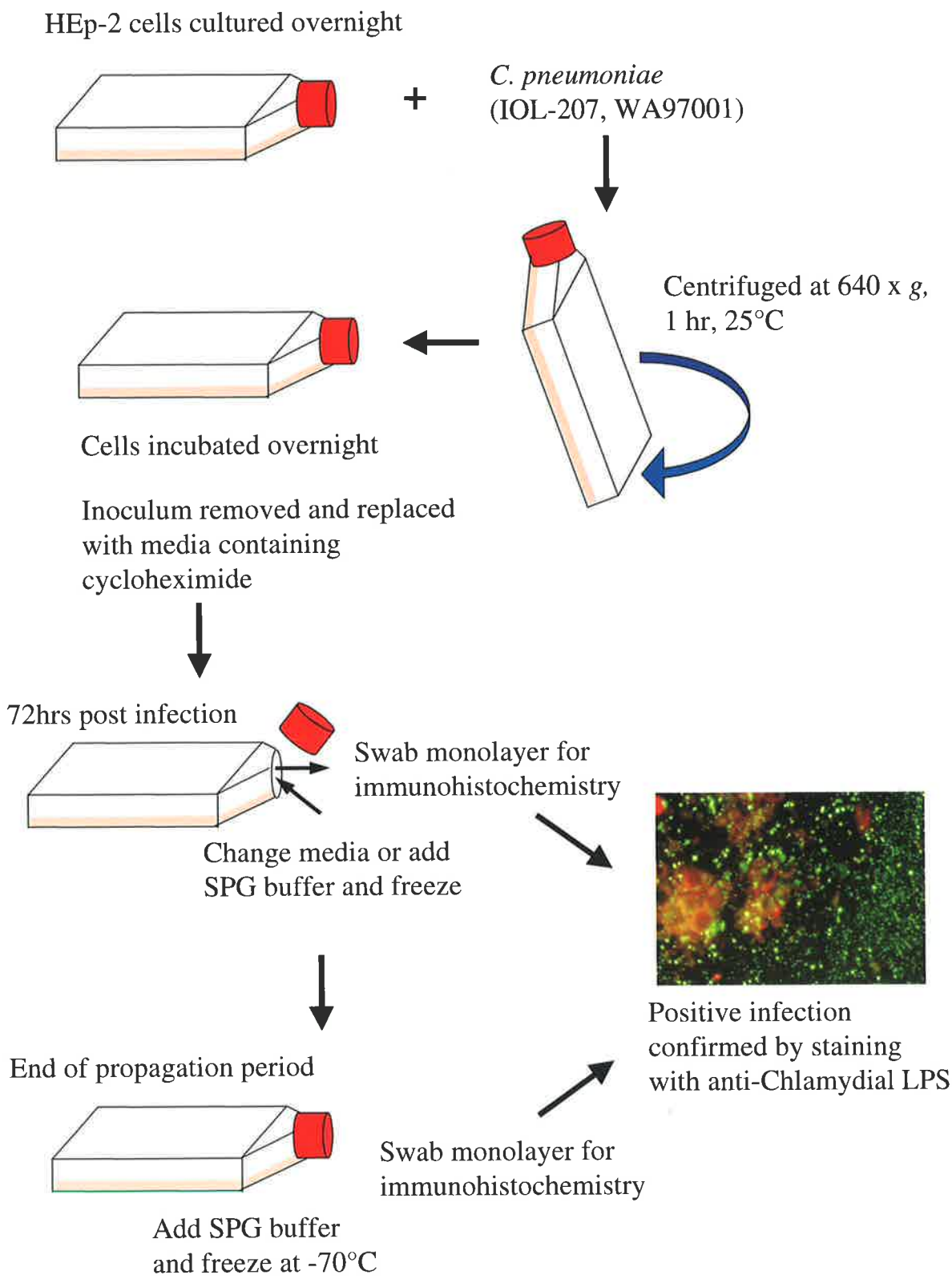


Figure 2.1: Propagation of *C. pneumoniae* in HEp-2 cells.
HEp-2 cells were cultured overnight in 75cm^2 flasks. The following day *C. pneumoniae* inoculum was added to the cells and flasks centrifuged for 1hr at 25°C before returning to the incubator overnight. Inoculum was discarded and replaced with complete DMEM containing 1µg/ml cycloheximide before allowing the cultures to grow until 72hrs post infection. The cell monolayer was swabbed to confirm infection by immunohistochemistry. If cultures were to continue growing, media was changed before culturing for a further 72hrs. At the end of the propagation period, SPG buffer was added to the cells and flasks were frozen at -70°C until harvesting.

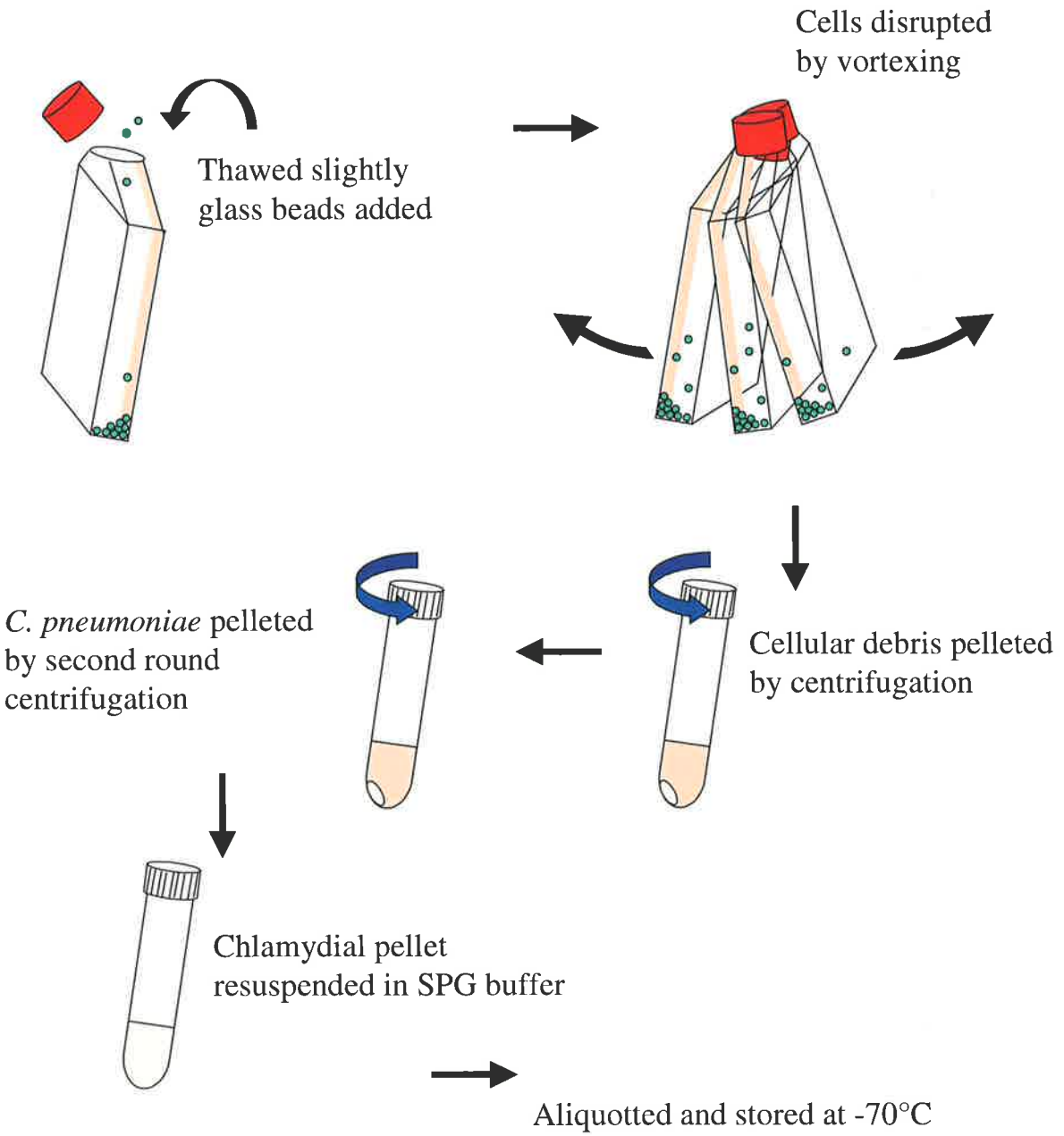


Figure 2.2: Harvesting of *C. pneumoniae* from infected HEp-2 cells.
Flasks which had been frozen at -70°C were thawed slightly under warm water and sterile glass beads added to the flask. Cells were mechanically disrupted by vigorously vortexing the flasks for 5 minutes. The contents of the flask were then transferred to tubes, which were centrifuged to pellet cellular debris. The supernatant was transferred to a second tube which underwent a second round of centrifugation to pellet *C. pneumoniae* organisms. The chlamydial pellet was then resuspended in SPG buffer, aliquotted and stored at -70°C until required.

Supernatant was transferred to a second centrifuge tube and centrifuged for a further 30 minutes at 30000 x *g* at 4°C before resuspending the chlamydial pellet in SPG buffer. Chlamydia harvested from different flasks were pooled and suspensions subsequently aliquotted and stored at -70°C until required. Quantitation of isolated *C. pneumoniae* was determined by inoculating fresh monolayers of HEp-2 cells with a thawed aliquot of *C. pneumoniae*.

Uninfected cell lysate preparation

Uninfected cell lysate was prepared to act as a negative control for subsequent experiments analysing the relationship between host cells and *C. pneumoniae*. Uninfected HEp-2 cells were prepared by the method described for infected HEp-2 cells but with the omission of chlamydial organisms from the inoculum. For example, after plating cells into flasks and culturing overnight, media was replaced with 5ml complete DMEM without *C. pneumoniae*. Uninfected cells were handled identical to infected cell cultures for the remainder of the infection period. Harvesting of uninfected cells was also the same as that of infected cells, where they were mechanically disrupted by vortexing with glass beads, before two rounds of centrifugation. Any remaining pellet was resuspended in SPG buffer. This preparation served as uninfected cell lysate in all subsequent experiments. To ensure that there was no contamination with *C. pneumoniae*, these preparations were screened for the presence of chlamydial DNA by polymerase chain reaction (described later).

C. pneumoniae quantitation assay

The standard method for quantitating chlamydial organisms is by determining the number of inclusion forming units (ifu) within a known volume of inoculum. These assays required plating HEp-2 cells in 24 well plates containing 10mm diameter glass coverslips at a cell density of $5x10^4$ cells/ml in complete DMEM and incubating overnight to allow cellular adherence. Media was then removed and replaced with 250µl of 1 in 5 or 1 in 10 serial

dilutions of *C. pneumoniae* in complete DMEM. Each dilution was added to cells in triplicate. Plates were centrifuged at 640 x *g*, for 1 hour at 25°C (Beckman GS-6R) before returning to the incubator overnight. The following day inoculum was replaced with 1ml complete medium plus 1μg/ml cycloheximide and cultured for a further 48 hours. At 72 hours post infection, media was removed and wells washed 3 times with 1 X PBS. Coverslips were then carefully removed from wells with forceps and adhered to glass microscope slides with DePeX mounting medium (BDH Chemicals, Kilsyth, VIC, Australia). Slides were left to air dry for a minimum of 1 hour. Slides were then fixed with acetone for 5 minutes before air drying and storing at -20°C until required for quantitative immunohistochemistry (discussed below). This allowed us to choose concentrations of *C. pneumoniae* comparable to other reported studies.

Confirmation of infection with C. pneumoniae

Successful infection of HEp-2 cells was verified by immunohistochemistry or polymerase chain reaction. Uninfected cell lysate was screened by polymerase chain reaction if there was suspected contamination by *C. pneumoniae* as indicated by immunohistochemistry.

Immunohistochemistry

Infection of HEp-2 cells with *C. pneumoniae* in 75cm^2 flasks was confirmed by taking a swab of each flask, 3 and 6 days post infection using sterile cotton wool buds and dabbing onto glass microscope slides. Swabs were air dried, followed by fixing in acetone for 5 minutes. Swabs were also taken from HEp-2 cells serving as uninfected controls.

Swabs from 3 and 6 day cultures, and coverslips from quantitation assays, were stained with a fluorescein isothiocyanate conjugated monoclonal antibody specific for chlamydial LPS, with Evans blue as a counter stain (CelLabs, Brookvale, NSW, Australia). After fixing, samples were incubated with chlamydial LPS antibody for 30 minutes at 37°C in a humidified

atmosphere to prevent slides drying out. Slides were then washed for 1 minute with 1 X PBS and coverslips applied with mounting media provided with the antibody. Slides were then sealed with clear nail varnish. Chlamydial organisms were then observed using a fluorescent microscope with a 490nm (absorption wavelength) filter (emission wavelength 530nm) at a final magnification of 400X (Olympus BX51, Olympus Corporation, Tokyo, Japan), see **Figure 2.3**.

The same method was performed on HEp-2 cell monolayers to be used for uninfected cell lysate preparations. If swabs from 3 or 6 day cultures of uninfected HEp-2 cells exhibited any positive staining for chlamydial LPS, the isolated uninfected cell lysate preparations were screened for *C. pneumoniae* DNA by polymerase chain reaction.

Enumeration of chlamydial inclusions was determined by assuming that an infected cell contained a single chlamydial inclusion, staining bright, apple-green with the FITC labelled chlamydial LPS antibody. The remainder of the cell, predominantly the nucleus, fluoresces red due to the autofluorescence of the Evans Blue counterstain. Infected cells were then counted by observing 20 random fields of view per coverslip and determining the number of infected and uninfected cells within that field. This resulted in approximately 500 cells being counted per coverslip. A typical set of slides from titration of a *C. pneumoniae* aliquot is shown in **Figure 2.4**. Batches of *C. pneumoniae* isolated from HEp-2 cells infected with the IOL-207 isolate on average yielded $1x10^8$ ifu per millilitre, and those infected with the WA97001 isolate on average yielded $1x10^6$ ifu per millilitre.

Susceptibility of airway epithelial cell lines to C. pneumoniae infection

Based on the methods described previously for propagation and harvesting of *C. pneumoniae*, we were able to successfully infect a variety of airway epithelial cell lines. The HEp-2 cell line is one of the most widely used and superior cell lines to maintain *C. pneumoniae*

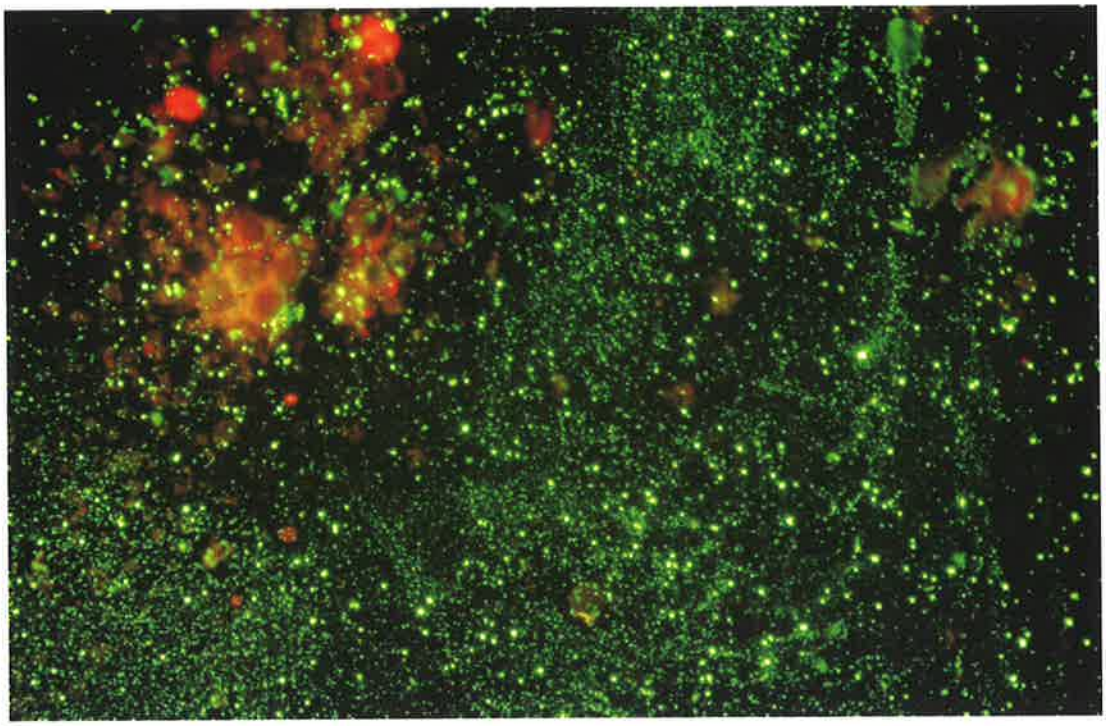


Figure 2.3: Swab of HEp-2 cell monolayer confirming infection with *C. pneumoniae*.
HEp-2 cells were cultured and infected as described in the methods section of the current chapter. The monolayer was then swabbed with a sterile cotton wool bud and dabbed onto a microscope slide, allowed to air dry and fixed with acetone. Slides were stained with a FITC conjugated anti-Chlamydial LPS antibody and the infection confirmed by visualisation under a fluorescent microscope with a 490nm filter at a final magnification of 400X. The bright, apple-green fluorescence is characteristic of a positive reaction between the antibody and chlamydial organisms (EB's and RB's). This is in contrast to the reddish brown colour of counterstained material.

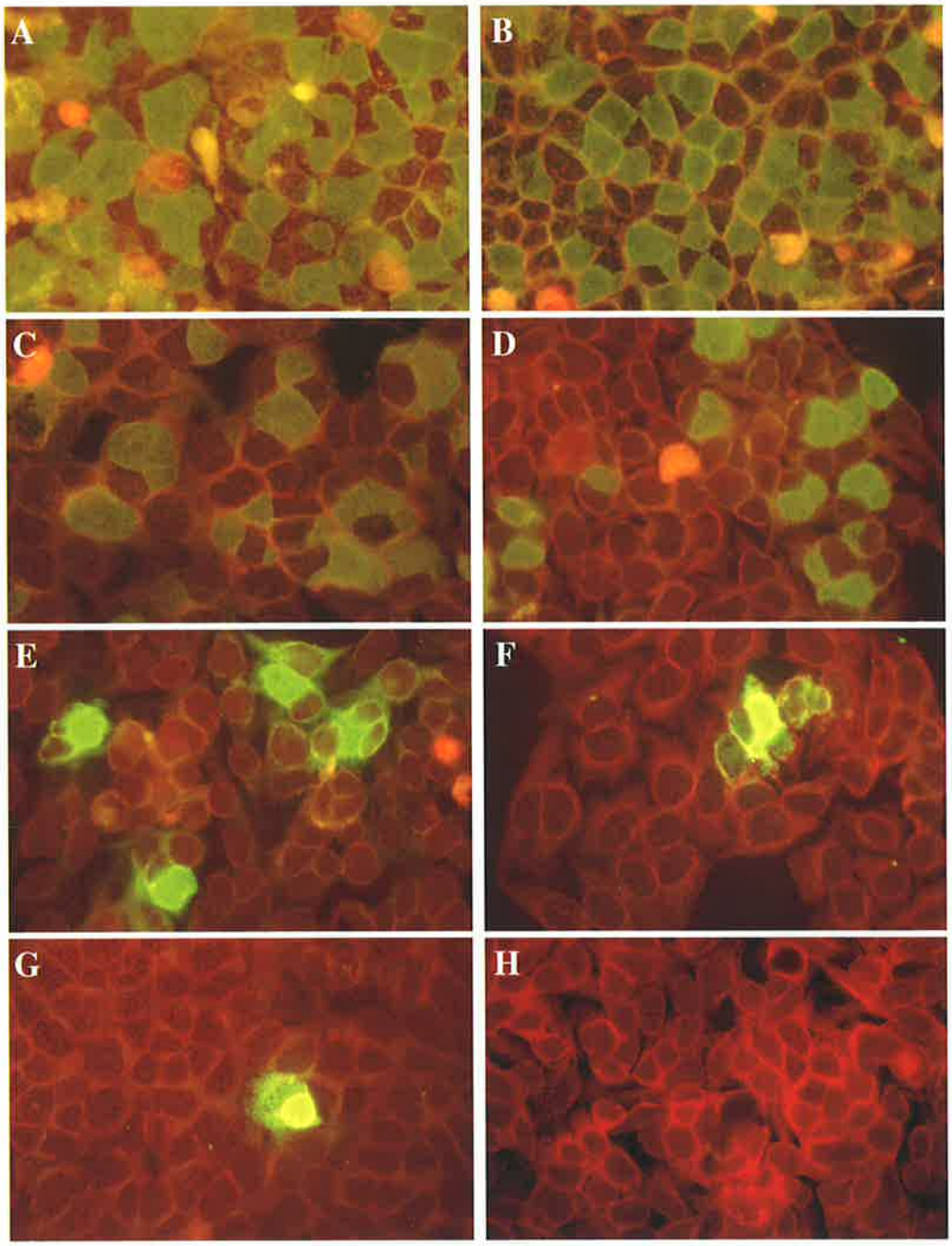


Figure 2.4: *C. pneumoniae* infected HEp-2 cells observed in quantification experiments.
HEp-2 cells were grown on coverslips in 24 well plates overnight before infecting with 1:10 serial dilutions of *C. pneumoniae*. Plates were centrifuged and left to incubate overnight before replacing inoculum with complete DMEM containing cycloheximide and cultured for 72 hours. Cells were then washed and coverslips air dried before fixing with acetone. Cells were stained with FITC conjugated anti-Chlamydial LPS and infected HEp-2 cells observed using a fluorescent microscope with a 490nm filter at a final magnification of 400X. **A**) HEp-2 cells infected with undiluted *C. pneumoniae* stock. **B-G**) HEp-2 cells infected with 1:10 serial dilutions of *C. pneumoniae*. **H**) Uninfected HEp-2 cells.

(143-145,387) and we were able to consistently show positive infection using this cell line (**Figure 2.5**).

We were also able successfully infect BEAS-2B cells with *C. pneumoniae*. Cells were plated into 24 well plates containing coverslips and infected as described above. Immuno-histochemistry was used to confirm infection 72 hours post infection and representative photomicrographs are depicted in **Figure 2.6**.

Two alternative airway epithelial cell lines 16HBE14o- and 16HAE14o- were also susceptible to *C. pneumoniae* infection. These cell lines were infected using the method to maintain *C. pneumoniae* in HEp-2 cells. The cells were plated into 75cm^2 tissue culture flasks and incubated overnight to allow attachment before removing media and replacing with 5ml complete media with or without *C. pneumoniae*. Flasks were centrifuged as described previously and incubated overnight after which the inoculum was replaced with complete media containing 1μg/ml cycloheximide. Cells were grown for a period of 7 days and media replaced on day 3. To confirm positive *C. pneumoniae* infection, cells were trypsinised and plated onto chamber slides and returned to the incubator for 4 hours to allow adherence. The media and slide chamber was then removed and slides allowed to air dry before fixing in acetone and slides stained for *C. pneumoniae* infection as described above. Both cell lines were susceptible to infection with *C. pneumoniae* as shown in **Figure 2.7**.

Polymerase Chain Reaction for detection of C. pneumoniae

C. pneumoniae infection of HEp-2 cells was also confirmed by using polymerase chain reaction to amplify the ompA gene from the *C. pneumoniae* genome. Primers were designed based on those described by Bodetti and Timms (13), **Table 2.1**. Reactions were performed in a total volume of 50μl using 2μl of *C. pneumoniae* or uninfected cell lysate preparation, boiled for 5 minutes and cooled on ice before addition to a PCR master mix. Final concentrations of reagents were as follows: 1.5mM $MgCl_2$, 1X PCR Buffer II, 1mM each of

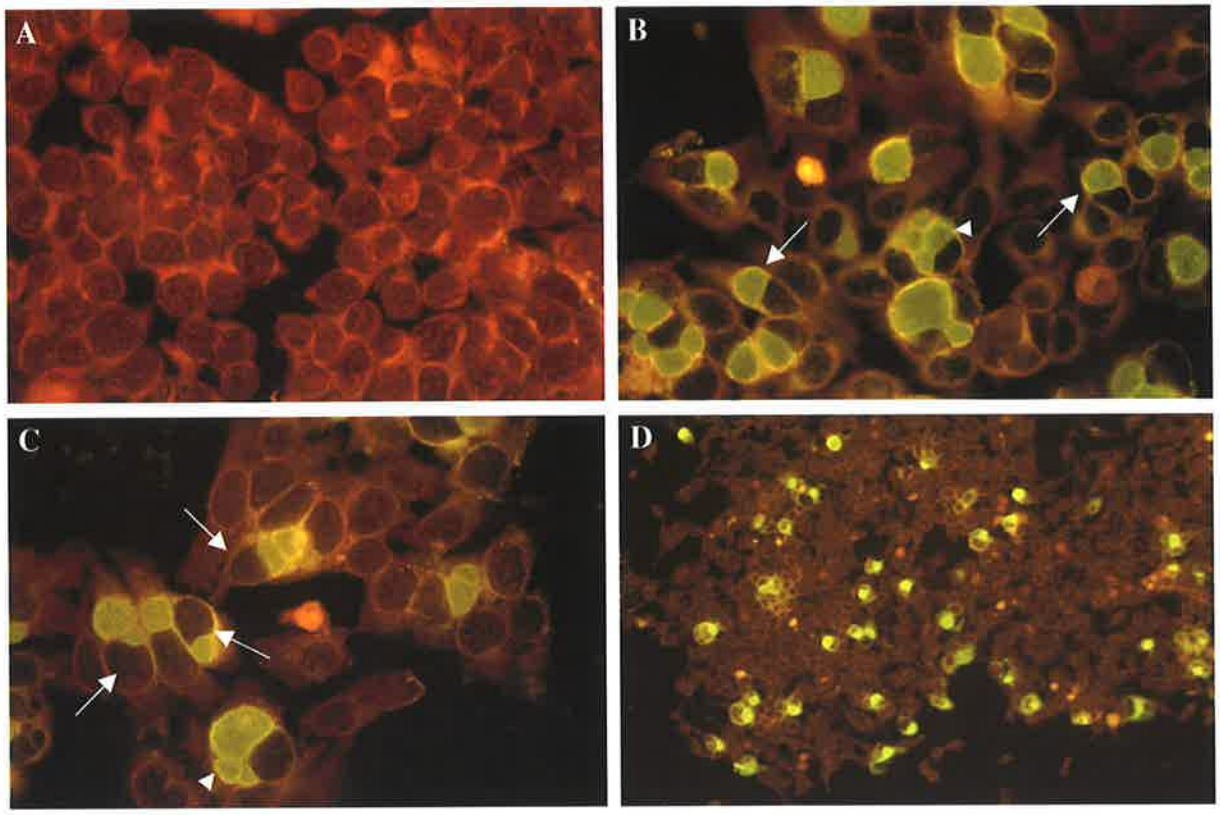


Figure 2.5: HEp-2 cells infected with *C. pneumoniae*.
HEp-2 cells were plated into 24 well plates containing coverslips and allowed to adhere overnight. Cells were then inoculated with *C. pneumoniae* and plates centrifuged for 1 hour before returning to the incubator overnight. Cell culture media was replaced with media containing cycloheximide and cells cultured until 72 hours post infection. Cells were washed with PBS before removing coverslips and mounting to microscope slides before allowing to air dry and fixed with acetone. Slides were then stained with a FITC conjugated anti-Chlamydial LPS antibody and infection observed using a fluorescent microscope with a 490nm filter. **A**) Uninfected HEp-2 cells, 400X magnification. **B**) and **C**) HEp-2 cells infected with *C. pneumoniae* (IOL-207) showing one inclusion per cell (arrows). HEp-2 cells infected with multiple chlamydial inclusions (arrowheads), 400X magnification. **D**) HEp-2 cells infected with *C. pneumoniae* isolate WA97001, 200X magnification.

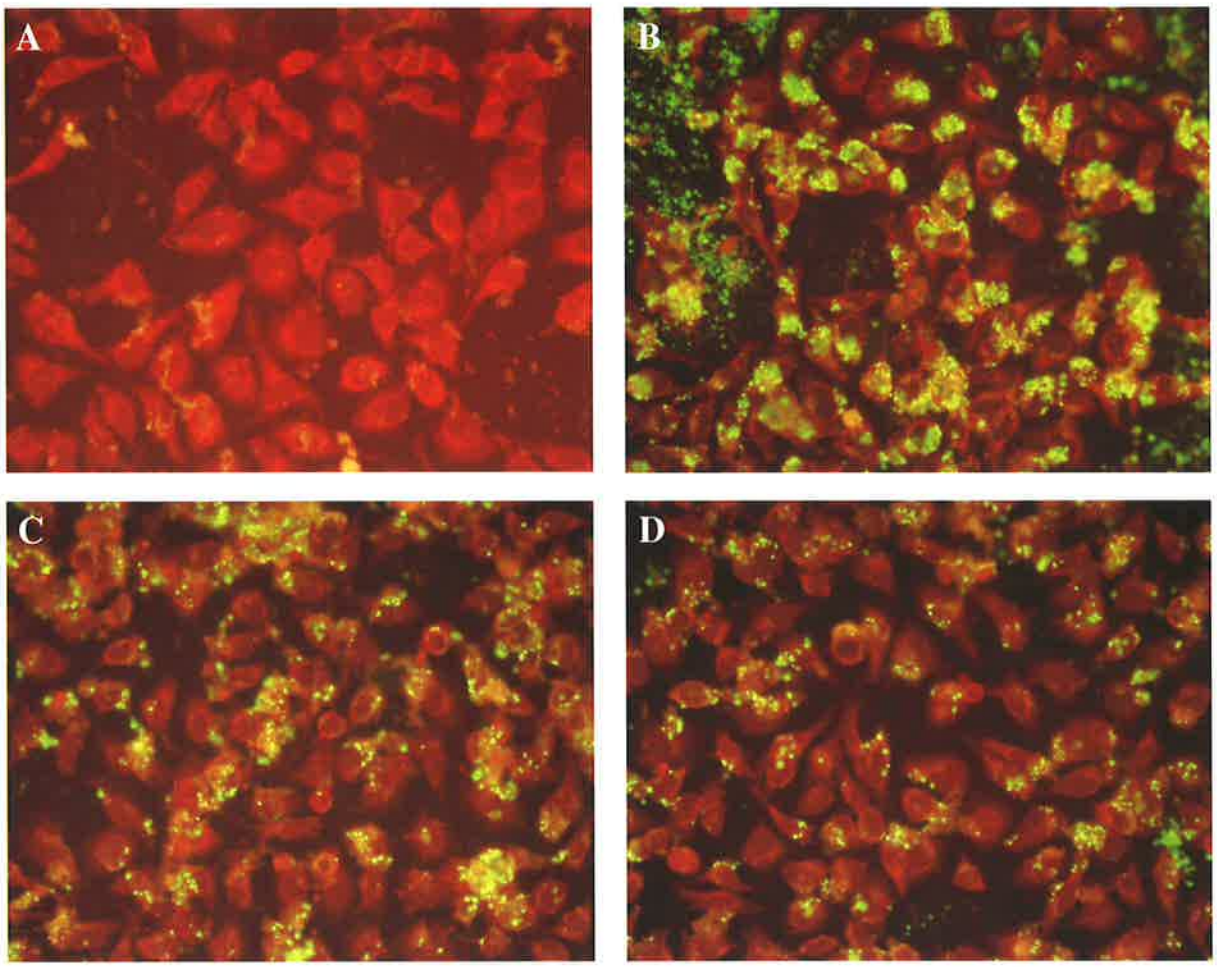


Figure 2.6: BEAS-2B cells infected with *C. pneumoniae*.
BEAS-2B cells were plated at $3x10^5$ cells/ml into 24 well plates containing coverslips and allowed to adhere overnight. The following day cells were inoculated with *C. pneumoniae* by centrifugation for 1 hour before returning to the incubator overnight. Cell media was replaced with media containing cycloheximide and cultured until 72 hours post infection. Cells were washed with PBS before removing coverslips and mounting to microscope slides before allowing to air dry and fixed with acetone. Slides were then stained with a FITC conjugated anti-Chlamydial LPS antibody and infection observed using a fluorescent microscope with a 490nm filter. **A**) Uninfected BEAS-2B cells. **B-D**) BEAS-2B cells infected with 1:10 serial dilutions of *C. pneumoniae* (IOL-207).

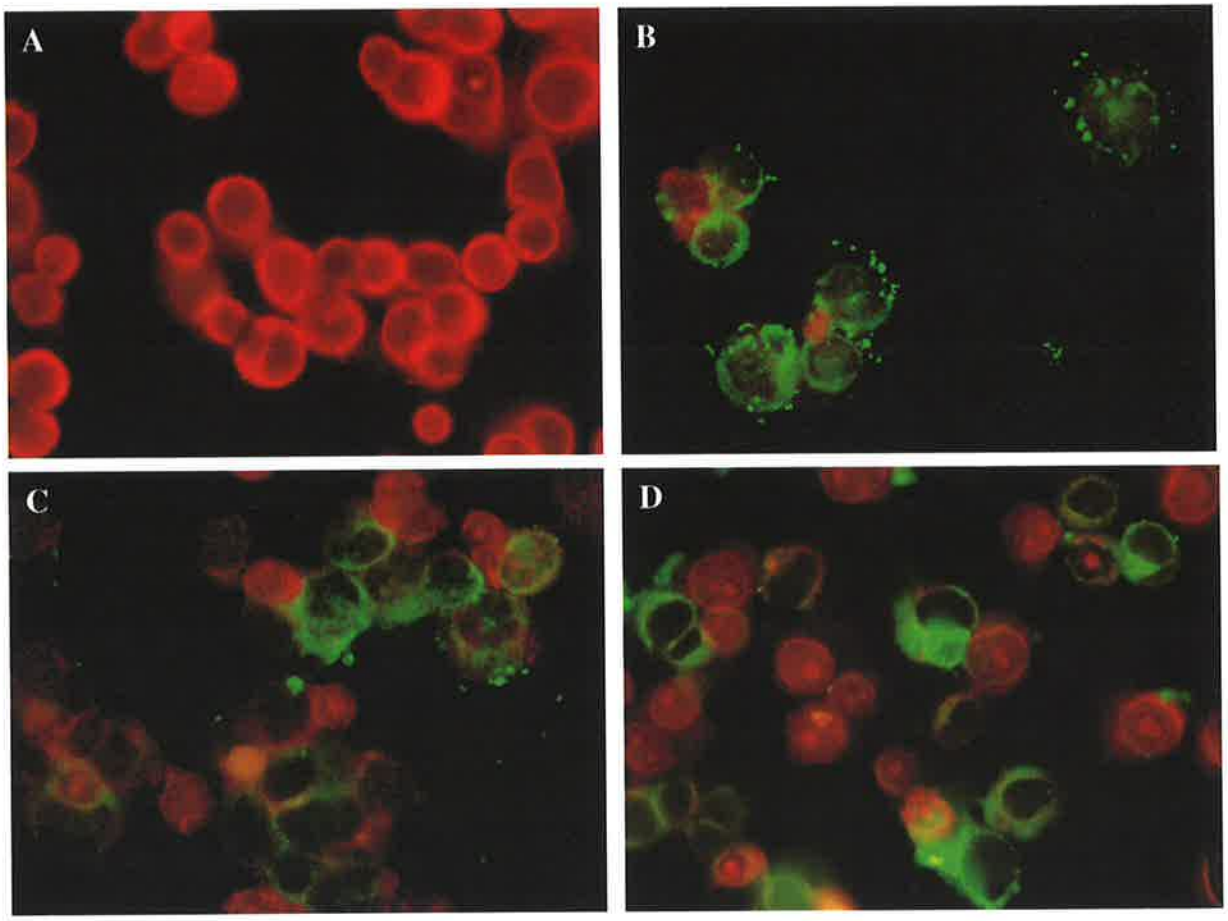


Figure 2.7: Airway epithelial cell lines susceptible to *C. pneumoniae* infection.
Cells were plated into 75cm^2 tissue culture flasks and allowed to adhere overnight before inoculation with *C. pneumoniae* (IOL-207). Flasks were centrifuged for 1 hour before returning to the incubator overnight. Cell culture media was replaced with media containing cycloheximide before allowing the cultures to grow for a total of 7 days with media changed on day 4. Cells were then trypsinised and plated into 8 well chamber slides, returned to incubate for 4 hours before media was removed and slides were air dried. Slides were fixed in acetone and stained with a FITC conjugated anti-Chlamydial LPS antibody. Slides were observed using a fluorescent microscope with a 490nm filter at a final magnification of 400X. **A**) Uninfected 16HBE14o- cells. **B**) 16HBE14o- cells infected with *C. pneumoniae*. **C**) 16HAEo- cells infected with *C. pneumoniae*. **D**) HEp-2 cells infected with *C. pneumoniae*.

deoxyribonucleoside triphosphates, 1μM 5' primer (Cpn5P), 1μM 3' primer (Cpn3P), 2.5 units AmpliTaq® DNA Polymerase and overlaid with paraffin oil. PCR was performed in a Perkin Elmer Thermal Cycler 480 with denaturation set at 95°C for 5 minutes, followed by 35 cycles of amplification at 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. Following the final amplification cycle, samples were cooled and held at 5°C. PCR products were analysed by gel electrophoresis. A typical representation of PCR detection of *C. pneumoniae* from infected HEp-2 cells, yielding a product size of 420bp is shown in **Figure 2.8**.

Target gene		Primer sequences	Product size
ompA	Cpn5P	cca ata tgc aca gtc caa acc taa aa	420bp
	Cpn3P	cta gat tta aac ttg ttg atc tga cag	

Table 2.1: Primer sequences for detection of *C. pneumoniae* by PCR.

Flow cytometry

Flow cytometry allows us to determine the physical and biochemical properties of single cells and is a well established technique in research applications. The studies documented here utilised flow cytometry to measure cytokine production and detection of molecules expressed on the surface of the cells of interest.

The current experiments utilised a FACSCalibur flow cytometer with an air-cooled 488nm argon laser. As cells pass through the argon laser beam, the light path is altered, dependent on the properties of the cell. Light diffracted to less than 10° of the incident light path of the laser, is detected and referred to as forward scatter (FSC). It provides information about the relative size of the cells passing through the laser beam. The relative complexity of the cell, including granularity, membrane irregularity or nuclear shape, is determined based on light reflected and diffracted from the incident light path and is detected at 90° to the laser beam,

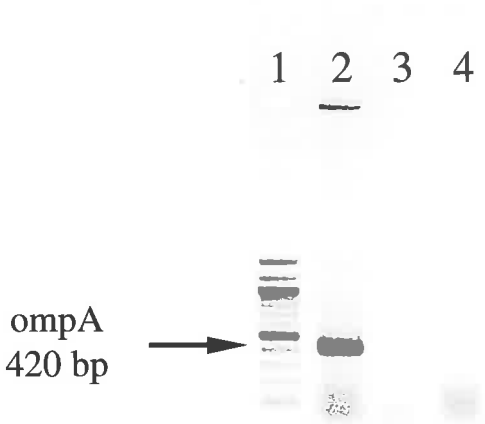


Figure 2.8: Screening of HEp-2 cell monolayers for *C. pneumoniae* by polymerase chain reaction.
HEp-2 cell monolayers were infected with *C. pneumoniae* before harvesting and purification of *C. pneumoniae* by centrifugation as described. Chlamydial pellet was resuspended in SPG buffer and an aliquot used for PCR detection of *C. pneumoniae*. Uninfected HEp-2 cells were prepared in a similar manner. Aliquots were boiled for 5 minutes before adding to the PCR mixture. PCR products were analysed by gel electrophoresis. **Lane 1**: 1kb DNA ladder. **Lane 2**: HEp-2 cells infected with *C. pneumoniae* (IOL-207). **Lane 3**: Negative control. **Lane 4**: Uninfected HEp-2 cells.

referred to as side scatter (SSC). The flow cytometer can also measure fluorescent parameters. The current studies utilised 3-colour or 3 fluorescent parameters, measured in fluorescent channels (FL)-1, 2 or 3. Fluorescent tags, or fluorochromes, conjugated to antibodies, absorb the blue light (488nm) of the argon laser and emit the energy at a different wavelength, specific to the fluorochrome. Light emitted in the green, orange and red regions of the spectrum are measured in FL-1, FL-2 and FL-3 respectively. The fluorochromes used in the current studies are shown in **Table 2.2**.

Fluorochrome	Absorbance wavelength	Emission wavelength	Region of spectrum
FITC	490nm	530nm	Green
PE	480nm	578nm	Orange
PE-Cy5	480nm	660-697nm	Red
PerCP	480nm	660-697nm	Red
7-AAD	480nm	670nm	Red

Table 2.2: Emission wavelength characteristics of fluorochromes used in flow cytometric analysis.

These optical signals are collected by a series of lenses, optical mirrors and filters, which enable the flow cytometer to separate the signals and convert them to electronic signals and digitises them for computer analysis. Data obtained from the flow cytometer was collected in list mode format and analysed using CellQuest software. Data can then be displayed in two main formats, dual or single parameter. Dual parameter display provides a graphic representation on an x,y grid of two different parameters on the same cell. Dual parameter displays include dot, contour and density plots. A single parameter display represents the frequency distribution of signal intensities observed for cells within a population and is displayed as a histogram.

Populations of cells can be selected by 'gating'. A 'gate' is a set of parameters used to define a subset of the cell population, for example FSC vs SSC. Other parameters, such as positive staining with fluorescent antibodies, can then be measured within the initial subset.

Evaluation of positive antibody staining is based on the non-specific binding of isotype control antibodies. The pattern of isotype control antibody binding in the dual parameter display is divided into rectangular quadrants by two perpendicular boundaries, termed quadrant markers. The quadrant markers are then set so that non-specific binding of isotype control antibodies is ≤ 2%. An isotype control antibody is an immunoglobulin of the same isotype as the monoclonal antibody of interest but without the specificity to any known human antigens.

Intracellular cytokine detection by flow cytometry

Cytokine expression by leucocytes in peripheral blood

Peripheral blood was diluted 1:1 with serum free DMEM with desired stimulus in 10ml polystyrene tubes (**Figure 2.9**). Blood was stimulated overnight with *C. pneumoniae* or a corresponding negative control consisting of blood incubated with uninfected cell lysate, to allow for the presence of HEp-2 cell remnants which may be present in the *C. pneumoniae* preparation. Positive controls consisted of *Escherichia coli* lipopolysaccharide (*E. coli* LPS; 100ng/ml), or the combination of phorbol myristate acetate (PMA; 6.25ng/ml) and ionomycin (ION; 1μg/ml), for cytokine production by monocytes and T lymphocytes respectively (all reagents were purchased from Sigma). The corresponding negative control for both cell types, was blood to which no additional stimulus was added (unstimulated). To allow intracellular cytokine detection, cells were incubated in the presence of Brefeldin A (10μg/ml; Sigma), a compound which interferes with the trafficking of cellular proteins from the endoplasmic reticulum to the Golgi apparatus. Protein secretion from the cell is therefore inhibited, thus retaining accumulated cytokines within the cytoplasm and allowing detection by flow cytometry (220,221,388). The following day, blood samples were incubated for 15 minutes at room temperature with 100μl EDTA (20mM) to aid the removal of cells which had adhered to the tubes overnight before transferring 500μl to flow cytometry tubes. Red blood cells were lysed by adding 2ml FACSlyse (BD Biosciences, San Jose, CA) to each flow tube

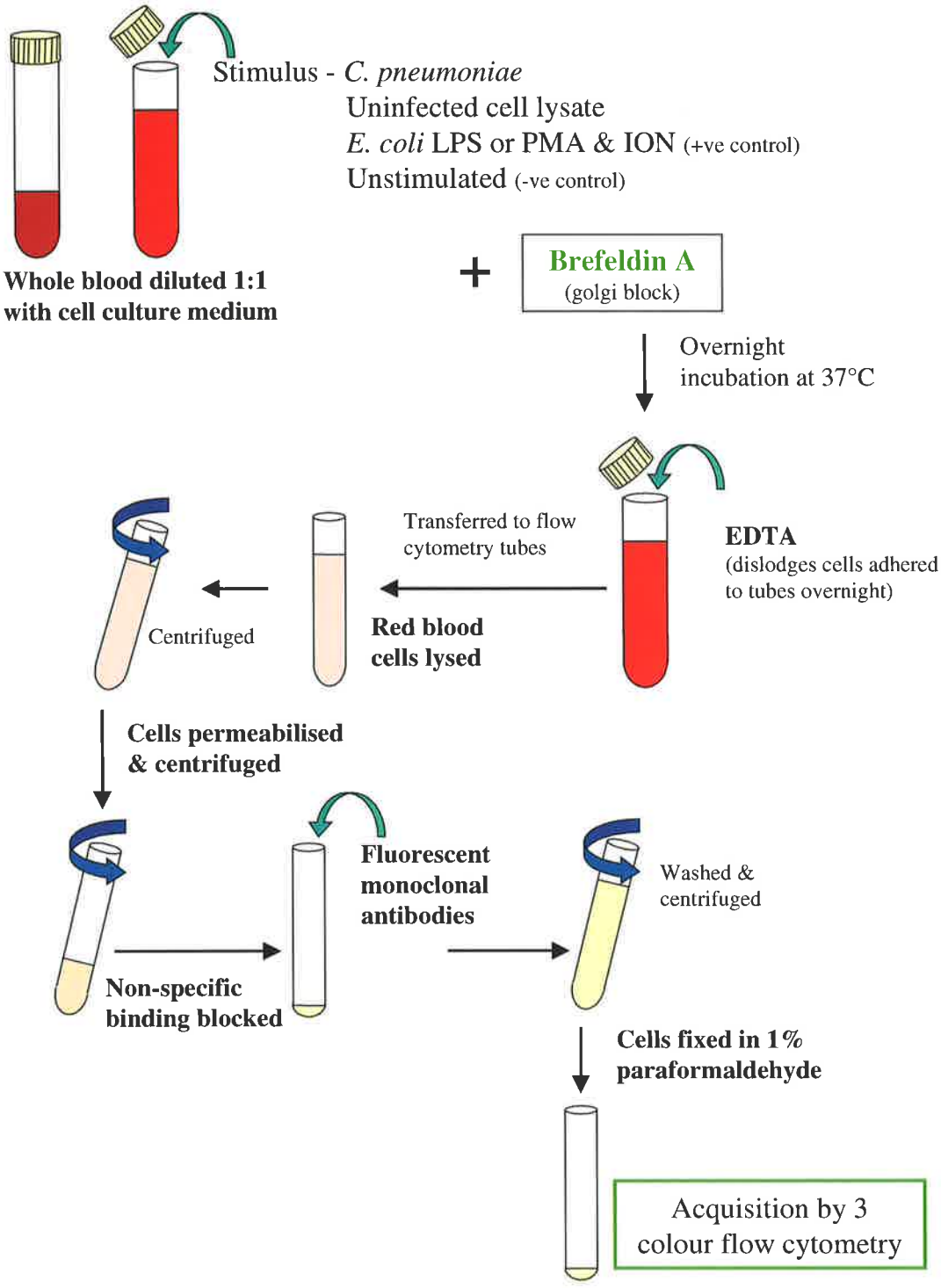


Figure 2.9: Preparation of whole blood for measurement of intracellular cytokine expression by flow cytometry. Whole blood was collected into tubes containing sodium heparin as an anticoagulant and blood diluted 1:1 with cell culture media. Blood was then stimulated overnight with the desired stimulus at 37°C in the presence of Brefeldin A (10μg/ml). The following day cells which may have adhered to tubes overnight were dislodged with EDTA (20mM) before transferring to flow cytometry tubes and red blood cells lysed with FACSlyse. Tubes were centrifuged and supernatant discarded before cells were permeabilised with FACSperm and non-specific binding blocked with Intragam. Anti-human monoclonal antibodies conjugated to fluorescent tags were added to flow tubes and incubated before washing and centrifuging. Supernatant was discarded before fixing cells in 1% paraformaldehyde and acquired by 3 colour flow cytometry using a Becton Dickinson FACSCalibur flow cytometer.

before mixing and incubating for 10 minutes at room temperature. Tubes were centrifuged for 1 minute at 4000rpm (Universal 32) and supernatant was decanted. Cells were permeabilised by adding 500µl FACSperm (BD Biosciences) to each tube and incubating for 10 minutes at room temperature. Cells were then washed with 2ml wash buffer (0.5% bovine serum albumin (BSA; Sigma) in Isoton II; Beckman Coulter, Gladesville, NSW, Australia) and centrifuged before decanting supernatant. Cells were then incubated for 20 minutes with 20µl Intragam (CSL Limited, Parkville, VIC, Australia) to block non-specific binding to F_c receptors. Cells were then incubated with 2.5µl each of fluorescent labelled monoclonal antibodies for 10 minutes at room temperature in the dark. Monocytes were identified with anti-CD14 PE-CY5 (Immunotech, Marseille, France) and T lymphocytes identified with anti-CD3 PE-CY5 (Immunotech). Intracellular cytokines were detected with a combination of fluorescein isothiocyanate (FITC) labelled monoclonal antibodies or phycoerythrin (PE) labelled monoclonal antibodies. Isotype control conjugated antibodies (BD Biosciences) were also used to determine non-specific background antibody binding. Cells were then washed with 2ml wash buffer, centrifuged and supernatant discarded. Cells were fixed in 30µl of 1% paraformaldehyde in Isoton II before acquisition. Three-colour flow cytometry was performed using a Becton Dickinson FACSCalibur flow cytometer. A minimum of 5000 (monocytes) or 10000 (T lymphocytes) gated events were acquired in list mode format and analysed using CellQuest software. Quadrant markers were set using the conjugated isotype control antibodies so that ≤ 2% of the cells exhibited non-specific binding (**Figure 2.10** and **2.11**).

In addition to preparing blood for flow cytometry, a cytospin of cells was prepared to confirm *C. pneumoniae* infection. Blood was lysed with FACSlyse, as described above and washed with wash buffer. Cells were then resuspended in 200µl of wash buffer before preparation of cytospins by centrifuging 50µl of the cell suspension onto microscope slides at 600rpm for 5 minutes using a cytocentrifuge (Cytospin 3, Shandon Incorporated, Pittsburgh, PA). Slides

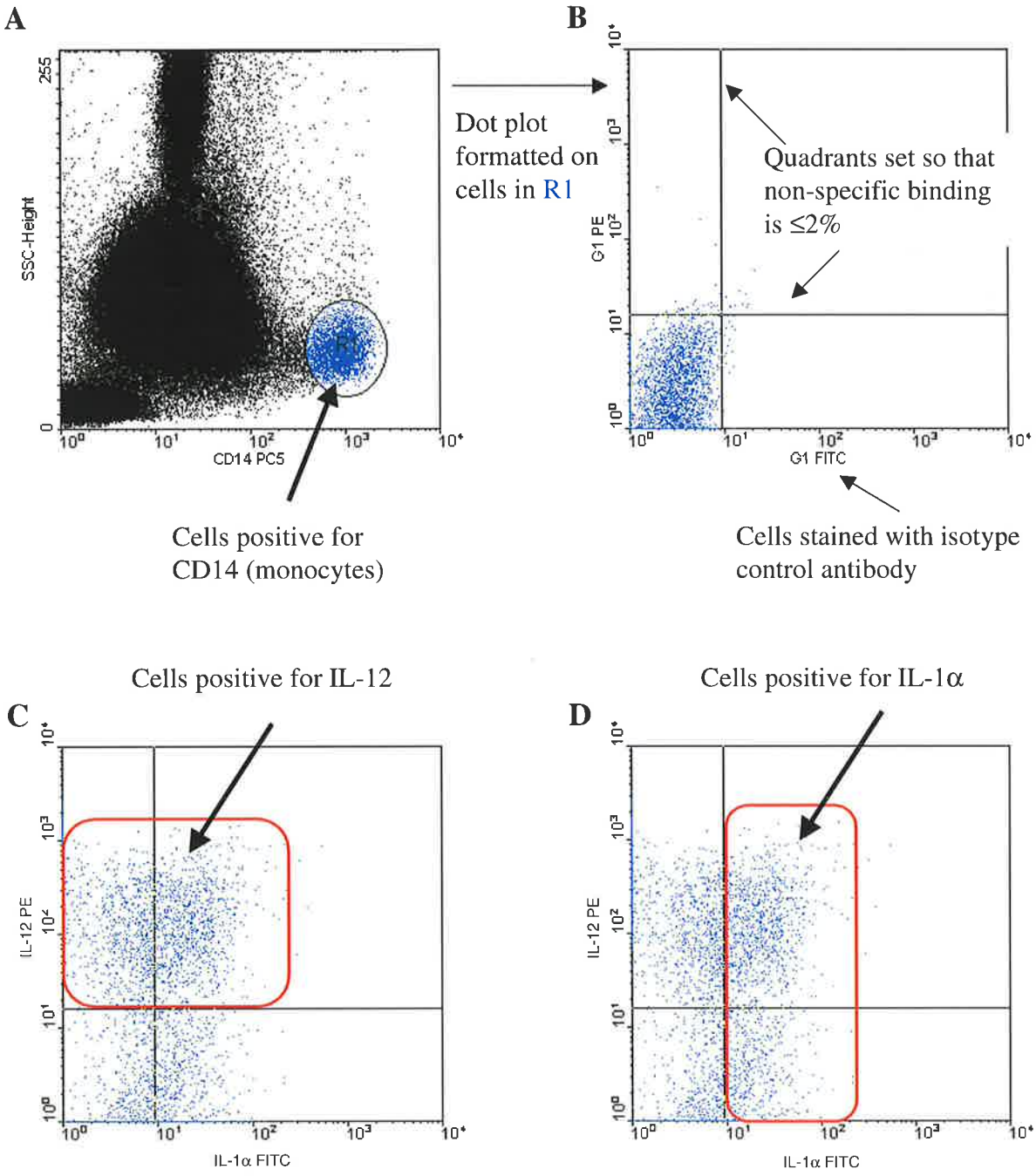


Figure 2.10: Flow cytometric analysis of intracellular cytokine expression in monocytes from whole blood. **A**) Whole blood stained with anti-CD14 PC5 antibody. Monocytes selected in Region 1 (R1) based on SSC and CD14 positive staining. **B**) Dot plot formatted on monocytes ($CD14^+$) in R1 and stained using an isotype control antibody, with quadrants set so that ≤2% of the cells exhibit non-specific binding. **C**) and **D**) Monocytes ($CD14^+$) stained with anti-human IL-12 PE and anti-human IL-1α FITC antibodies. Circles (red) depict cells expressing IL-12 (**C**) or IL-1α (**D**). Cells in upper right quadrants in dot plots (**C**) and (**D**) co-express both IL-12 and IL-1α.

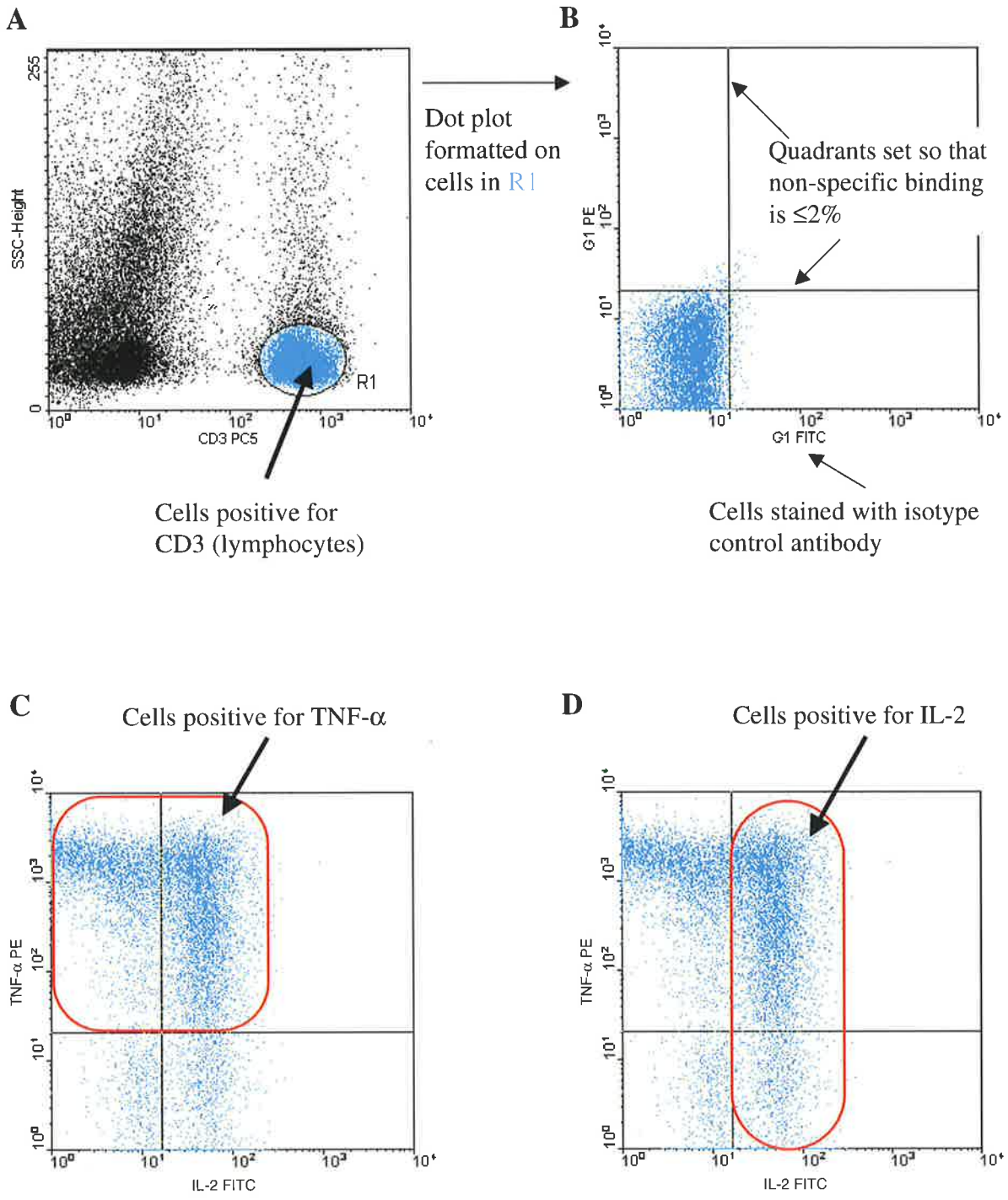


Figure 2.11: Flow cytometric analysis of intracellular cytokine expression in lymphocytes from whole blood. **A**) Whole blood stained with anti-CD3 PC5 antibody. Lymphocytes selected in Region 1 (R1) based on SSC and CD3 positive staining. **B**) Dot plot formatted on lymphocytes ($CD3^+$) in R1 and stained using an isotype control antibody, with quadrants set so that ≤2% of the cells exhibit non-specific binding. **C**) and **D**) Lymphocytes ($CD3^+$) stained with anti-human TNF-α PE and anti-human IL-2 FITC antibodies. Circles (red) depict cells expressing TNF-α (**C**) or IL-2 (**D**). Cells in upper right quadrants in dot plots (**C**) and (**D**) co-express both TNF-α and IL-2.

were air dried and fixed for 5 minutes in acetone prior to immunohistochemical staining for detection of *C. pneumoniae* as described previously (**Figure 2.12**).

Extracellular cytokine production detected by flow cytometry

Airway epithelial cells

The release of various cytokines into the supernatant of *C. pneumoniae* stimulated BEAS-2B cells were analysed using a Human Inflammation Cytometric Bead Array kit (BD Biosciences). BEAS-2B cells were plated into 24 well tissue culture plates at a density of $3x10^5$ cells/ml in complete K-SFM and incubated overnight to allow adherence to the plates. The following day media was replaced with fresh media containing the desired stimulus in a total volume of 1ml. Stimulus included *C. pneumoniae*, uninfected cell lysate as a negative control, as a positive control TNF-α (20 ng/ml; Promega Corporation, Madison, WI) and unstimulated cells to which no additional stimulus was added served as a negative control. Cells were then incubated overnight at 37°C in a humidified atmosphere containing 95% O_2 and 5% CO_2. The following day supernatant was removed from the cells and transferred to 1.5ml microcentrifuge tubes and stored at -70°C until ready to be assayed.

Cytokine production by BEAS-2B cells was measured following the protocol for culture supernatant provided by the manufacturer. Standards were reconstituted and serially diluted with assay diluent providing standard curves for each cytokine (IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70) from approximately 5000pg/ml to 20pg/ml. Capture beads were mixed in equal proportions before adding to appropriate assay tubes. An equal volume of the Human Inflammation PE Detection Reagent was then added to all tubes before adding either cell culture supernatant or prepared standard to appropriate tubes. The tubes were then incubated for 3 hours at room temperature in the dark. After this time, tubes were washed with 1ml of Wash Buffer (provided in kit) and centrifuging tubes at 200 x *g* for 5 minutes. The supernatant was carefully aspirated and discarded before resuspending the bead pellet in

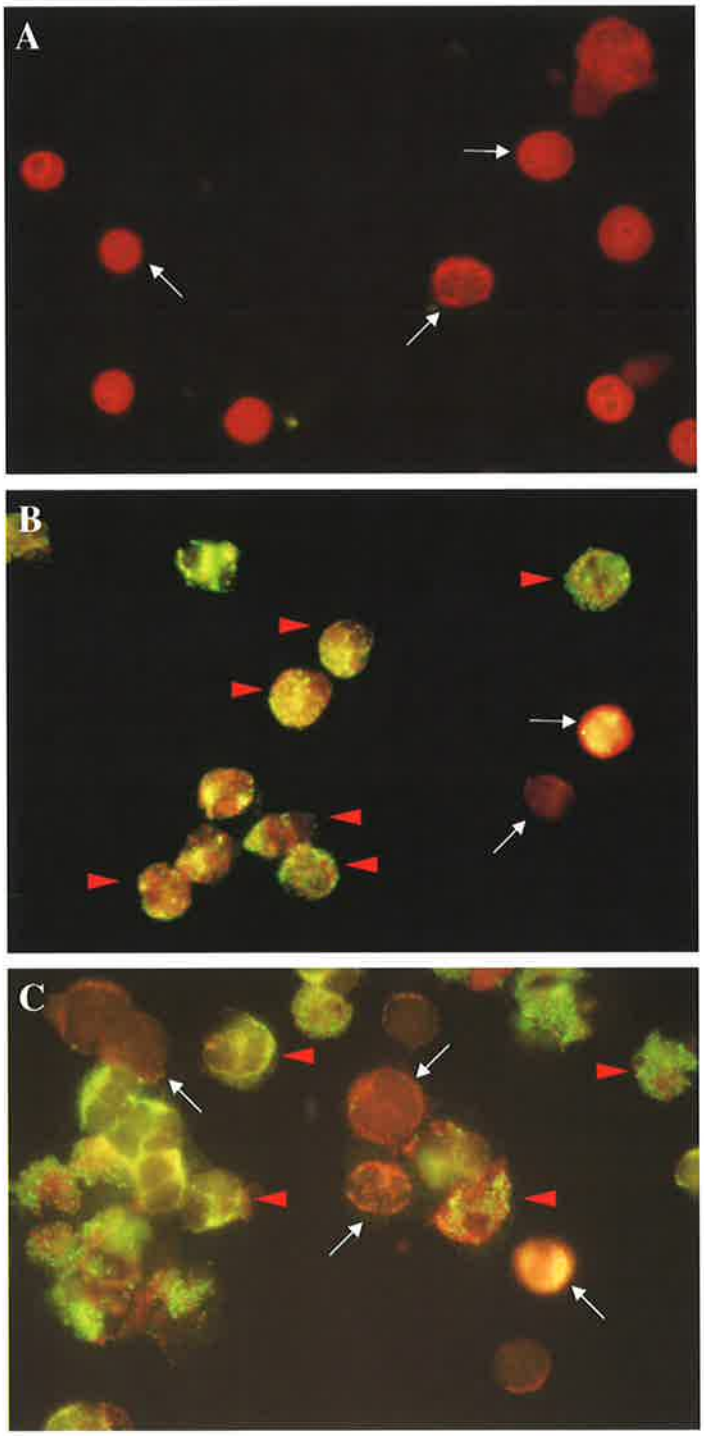


Figure 2.12: Whole blood leucocytes infected with *C. pneumoniae.*
Whole blood was diluted 1:1 with cell culture media with or without *C. pneumoniae* and incubated overnight. Red blood cells were lysed with FACSlyse, centrifuged for 1 minute at 4000rpm (Universal 32) before washing with wash buffer and centrifuged. A cytospin of cells was prepared and allowed to air dry before fixing in acetone. Slides were stained with a FITC conjugated anti-Chlamydial LPS antibody and the infection confirmed by visualisation under a fluorescent microscope with a 490nm filter at a final magnification of 1000X under oil. **A**) Uninfected leucocytes. **B**) Leucocytes infected with *C. pneumoniae* (IOL-207). **C**) Leucocytes infected with *C. pneumoniae* (WA97001). White arrows indicate uninfected cells. Red arrowheads indicate leucocytes infected with *C. pneumoniae.*

300µl of Wash Buffer. Samples were then acquired using a Becton Dickinson FACSCalibur flow cytometer and samples analysed using BD CBA Software. According to the instruction manual included with this kit, the sensitivity of this assay allows accurate detection of cytokines released into cell supernatant within the range of 20 pg/ml up to 5000 pg/ml. However, the following concentrations are provided by the manufacturer as the level of sensitivity for each of the six proteins measured: IL-8, 3.6 pg/ml; IL-1β, 7.2 pg/ml; IL-6, 2.5 pg/ml; IL-10, 3.3 pg/ml; TNF, 3.7 pg/ml; and IL-12p70, 1.9 pg/ml. With regard to the specificity of this assay, there is no reported cross reactivity or background detection of protein in other Capture Bead populations using this assay and the antibody pairs have been screened for specific reactivity with their specific proteins.

Measurement of surface marker expression by flow cytometry

Peripheral blood

Peripheral blood was diluted 1:1 with serum free DMEM with desired stimulus in 10ml polystyrene tubes (**Figure 2.13**). Blood was stimulated overnight with *C. pneumoniae* preparation or a corresponding negative control consisting of blood incubated with uninfected cell lysate, to allow for the presence of HEp-2 cell remnants which may be present in the *C. pneumoniae* preparation. Positive controls consisted of *E. coli* LPS (100ng/ml) stimulated blood for monocytes and neutrophils or phytohaemagglutinin (PHA; 2µg/ml; Sigma) stimulated blood for T lymphocytes. The corresponding negative control for all cell types was blood to which no additional stimulus was added (unstimulated).

The following day, blood samples were incubated for 15 minutes at room temperature with 100µl EDTA (20mM) to aid the removal of cells which had adhered to the tubes overnight, and 20µl Intragam (CSL) to block F_c receptors. The expression of surface molecules were detected with a combination of FITC labelled monoclonal antibodies or PE labelled monoclonal antibodies (3µl), which were added directly to flow cytometry tubes before

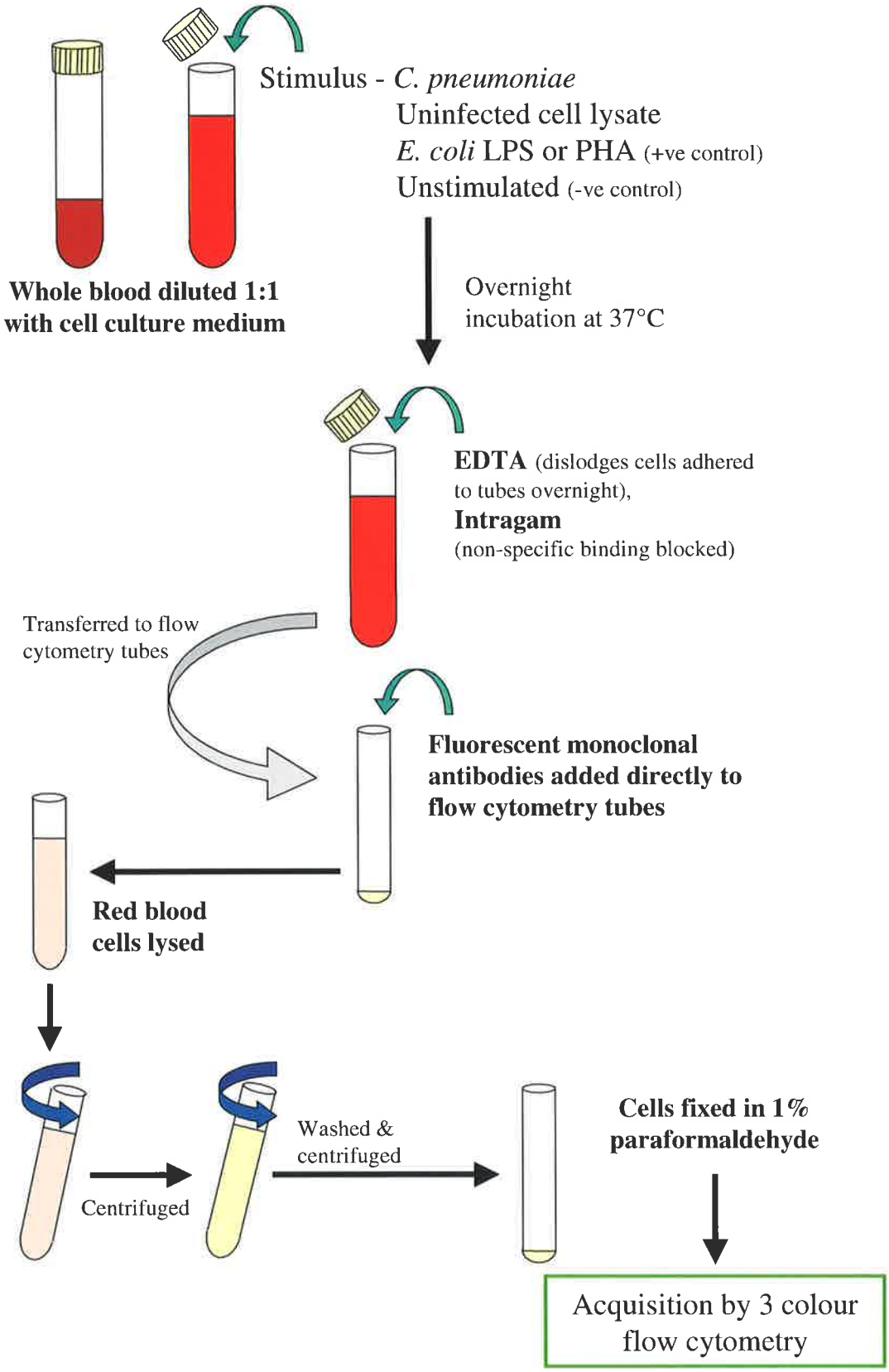


Figure 2.13: Preparation of whole blood for measurement of cell surface marker expression by flow cytometry. Whole blood was collected into tubes containing sodium heparin as an anticoagulant and blood diluted 1:1 with cell culture media. Whole blood was then stimulated overnight with the desired stimulus at 37°C. The following day cells which may have adhered to tubes overnight were dislodged with EDTA (20mM) and non-specific binding blocked with Intragam. The blood mixture was transferred to flow cytometry tubes, already containing anti-human monoclonal antibodies conjugated to fluorescent tags. Red blood cells were lysed with FACSlyse and tubes centrifuged before discarding supernatant. Cells were then washed with wash buffer, centrifuged and supernatant discarded before fixing cells in 1% paraformaldehyde. Cells were then acquired by 3 colour flow cytometry using a Becton Dickinson FACSCalibur flow cytometer.

transferring 300µl of the blood mixture to each tube and incubated for 10 minutes at room temperature in the dark. Isotype control conjugated antibodies (BD Biosciences) were used to determine non-specific background antibody binding. Monocytes were identified with anti CD14-PE-CY5 (Immunotech) and T lymphocytes identified with anti-CD3 PE-CY5 (Immunotech). Neutrophils were also identified based on anti-CD14 PE-CY5 staining, together with forward and side scatter characteristics. Neutrophils exhibit positive CD14 staining to a lesser extent than monocytes. Red blood cells were lysed by adding 2ml FACSlyse (BD Biosciences) to each flow tube before mixing and incubating for 10 minutes at room temperature. Tubes were centrifuged for 1 minute at 4000rpm (Universal 32) and supernatant was decanted. Cells were then washed with 2ml wash buffer (0.5% BSA in Isoton II) and centrifuged before decanting supernatant. Cells were then fixed in 30µl of 1% paraformaldehyde in Isoton II before acquisition. Three-colour flow cytometry was performed using a Becton Dickinson FACSCalibur flow cytometer. A minimum of 5000 (monocytes or neutrophils) or 10000 (T lymphocytes) gated events were acquired in list mode format and analysed using CellQuest software. Quadrant markers were set using the conjugated isotype control antibodies so that ≤ 2% of the cells exhibited non specific binding (**Figure 2.14 – 2.16**).

Airway epithelial cells

Airway epithelial cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate or 20ng/ml TNF-α (Promega Corporation) in 10ml polystyrene tubes at a concentration of $1x10^6$ cells/ml in a total volume of 2ml (**Figure 2.17**). The following day cells were gently resuspended using a tuberculin syringe (Terumo Medical Corporation, Elkton, MD) and 200µl transferred into flow cytometry tubes before centrifuging for 1 minute at 4000rpm (Universal 32) to remove supernatant. Non-specific binding was blocked by incubating the cells with 20µl Intragam for 20 minutes at room temperature. Surface marker expression of adhesion, antigen presentation and costimulatory molecules was then detected by incubating

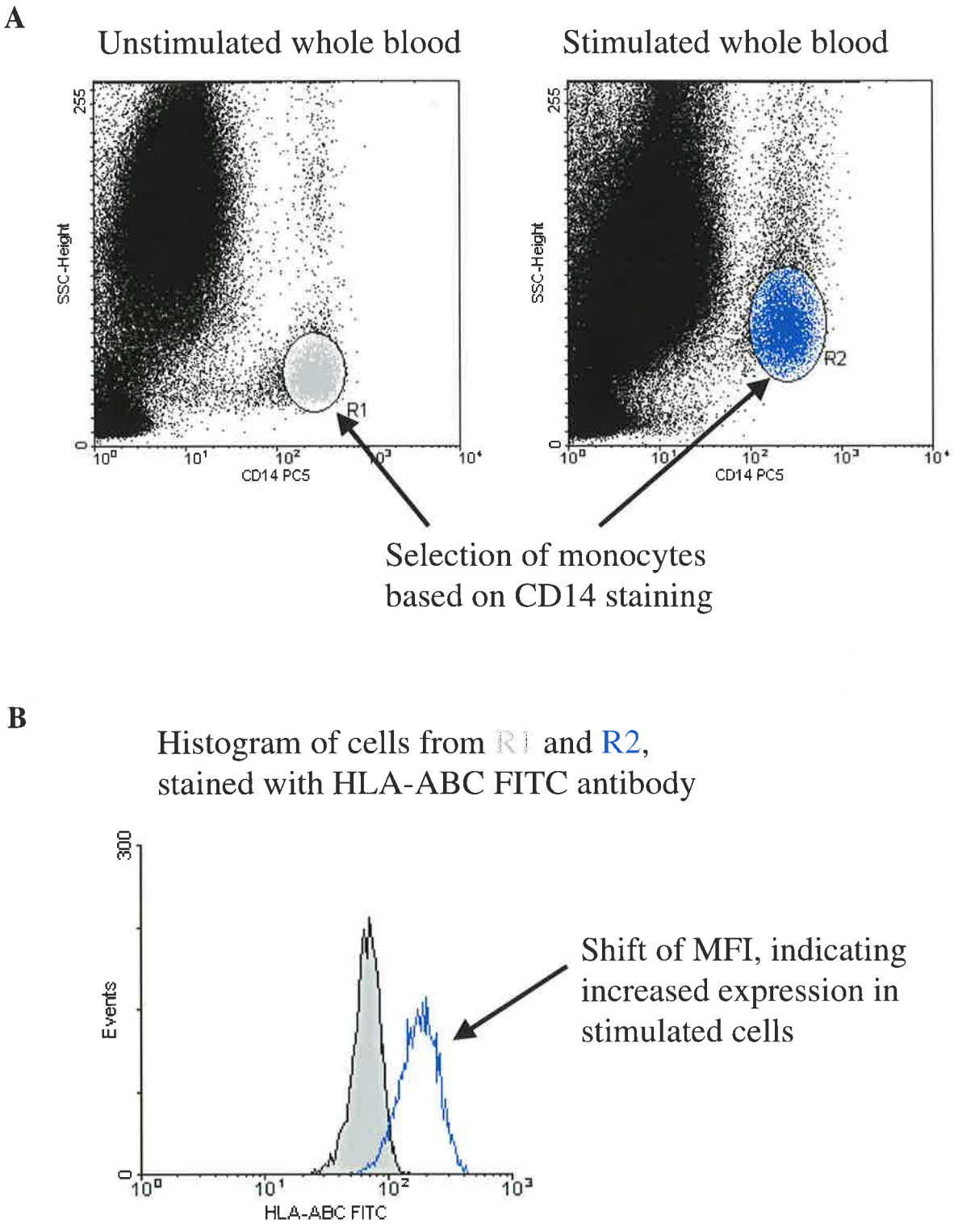


Figure 2.14: Example of flow cytometric analysis of surface marker expression by whole blood monocytes. **A**) Dot plots showing selection of monocytes from unstimulated or stimulated whole blood, based on CD14 staining and SSC characteristics. **B**) Histogram of $CD14^+$ cells, selected from dot plots in (**A**), showing mean fluorescent intensity (MFI) of HLA-ABC surface marker expression. Note the shift of the MFI curve to the right demonstrating increased expression by stimulated cells.

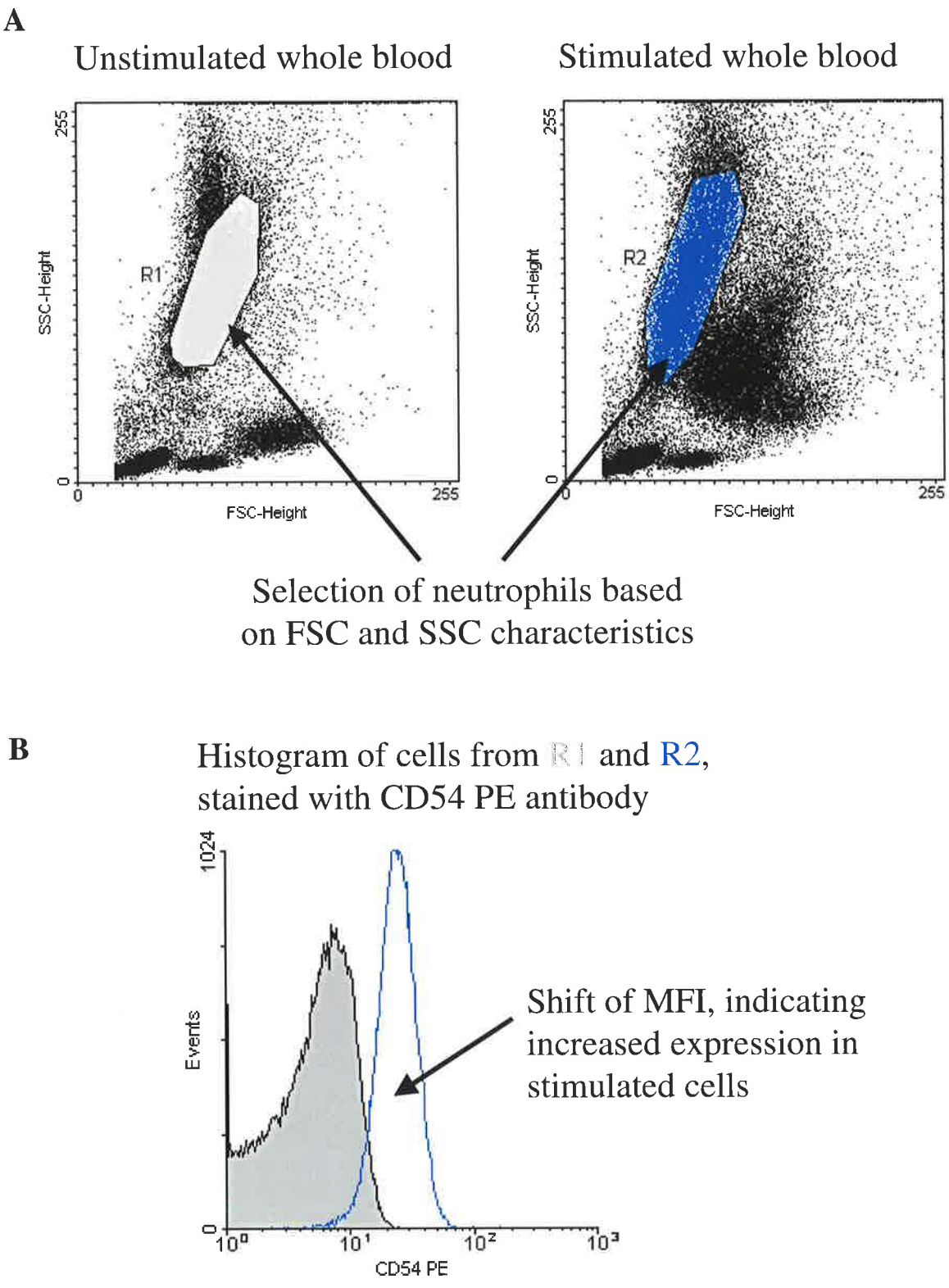


Figure 2.15: Example of flow cytometric analysis of surface marker expression by whole blood neutrophils. **A**) Dot plots showing selection of neutrophils from unstimulated or stimulated whole blood, based on FSC and SSC characteristics. **B**) Histogram of selected cells, from regions defined in dot plots in (**A**), showing mean fluorescent intensity (MFI) of CD54 surface marker expression. Note the shift of the MFI curve to the right demonstrating increased expression by stimulated cells.

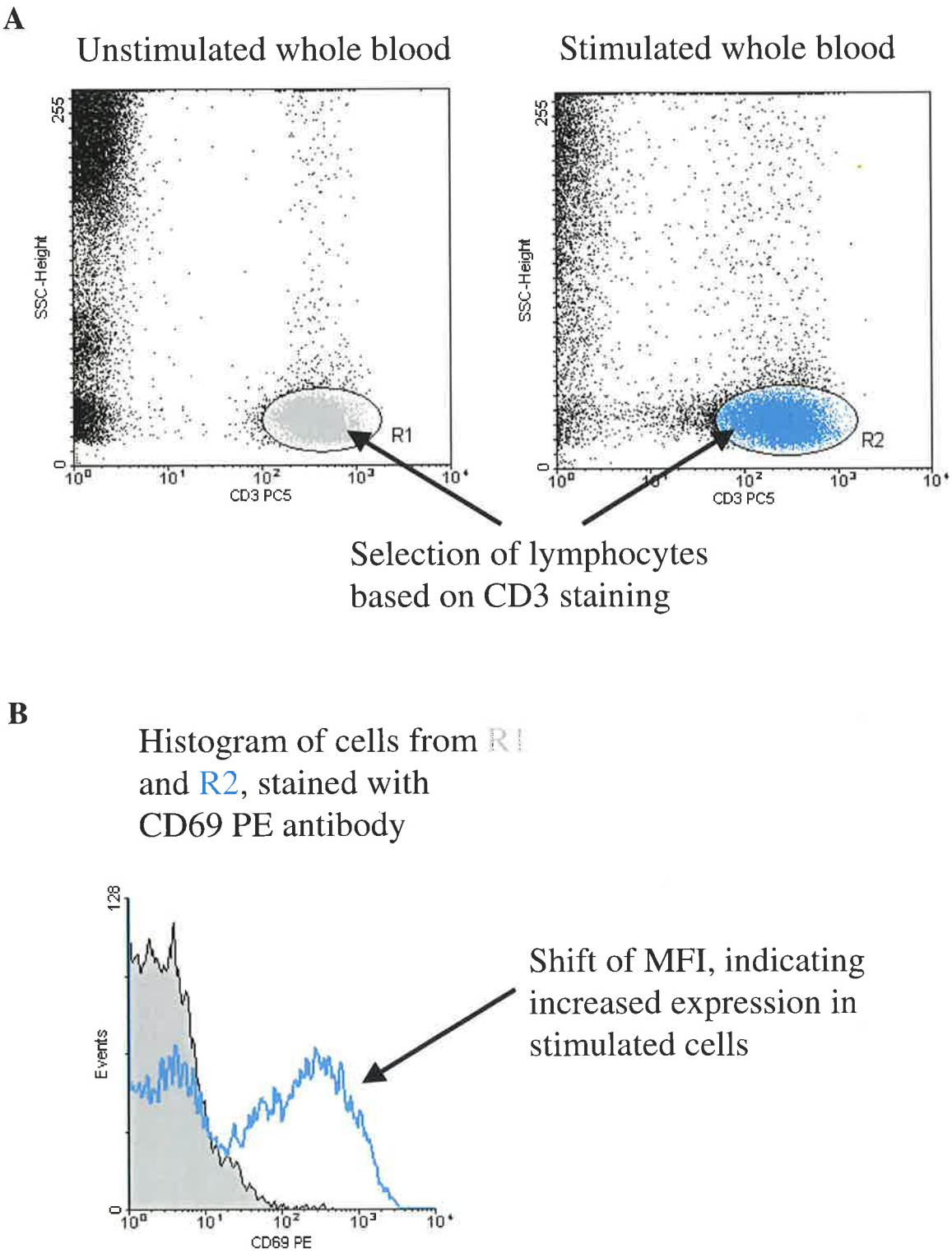


Figure 2.16: Example of flow cytometric analysis of surface marker expression by whole blood lymphocytes. **A**) Dot plots showing selection of lymphocytes from unstimulated or stimulated whole blood, based on CD3 staining and SSC characteristics. **B**) Histogram of $CD3^+$ cells, selected from dot plots in (**A**), showing mean fluorescent intensity (MFI) of CD69 surface marker expression. Note the shift of the MFI curve to the right demonstrating increased expression by stimulated cells.

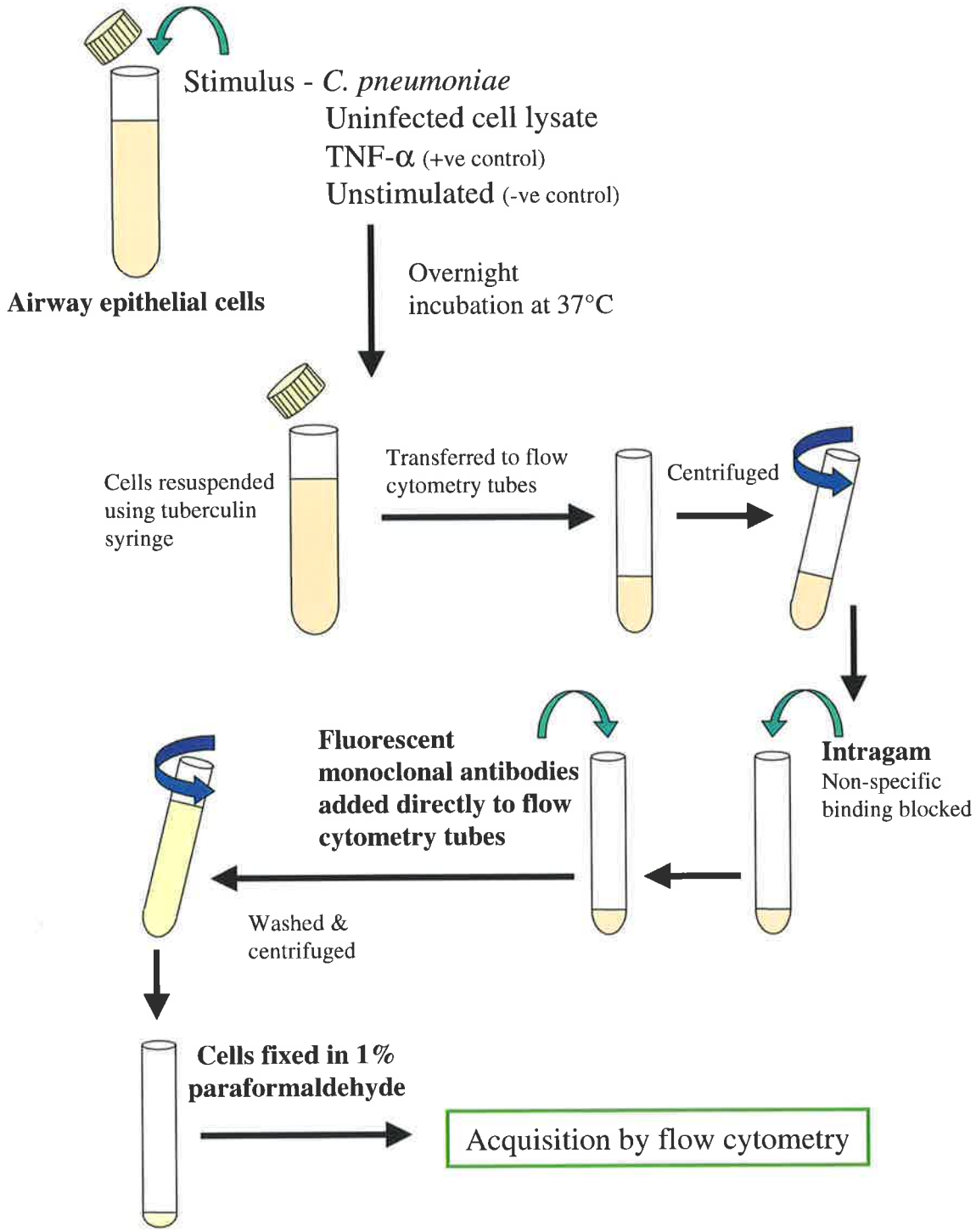


Figure 2.17: Preparation of airway epithelial cells for measurement of cell surface marker expression by flow cytometry.
Airway epithelial cells were stimulated overnight with the desired stimulus at 37°C. The following day, cells were resuspended using a tuberculin syringe before transferring to flow cytometry tubes. Cells were centrifuged to remove supernatant and non-specific binding blocked with Intragam. Monoclonal antibodies conjugated to fluorescent tags were added to the cells and allowed to incubate before washing and centrifuging. Wash buffer was discarded and cells fixed by resuspending in 1% paraformaldehyde. Cells were then acquired by flow cytometry using a Becton Dickinson FACSCalibur flow cytometer.

temperature in the dark. Cells were then washed with 2ml wash buffer and centrifuged for 1 minute at 4000rpm (Universal 32). Wash buffer was discarded and cells resuspended in 30µl wash buffer before acquisition. Two colour flow cytometry was performed using a Becton Dickinson FACSCalibur flow cytometer (**Figure 2.18**). A minimum of 20000 events were acquired in list mode format and analysed using CellQuest software.

Measurement of total cell apoptosis by flow cytometry

Monocytes

After incubation of blood overnight with desired stimulus, (described earlier) 100µl of EDTA and 20µl Intragam was added to each tube, vortexed and incubated for 15 minutes at room temperature (**Figure 2.19**). During this time, 3µl of anti-CD14 FITC (BD Biosciences) was added to each flow cytometry tube. After vortexing the blood briefly, 500µl of blood was transferred to each flow tube, already containing anti-CD14 FITC and incubated for a further 10 minutes at room temperature in the dark. Red blood cells were lysed by adding 2ml Red Blood Cell Lysis Buffer (Sigma) and incubating for a further 10 minutes at room temperature. Tubes were then centrifuged for 1 minute at 4000rpm (Universal 32) and supernatant decanted. Cells were then washed in 2ml wash buffer (twice if deemed necessary) and centrifuged for 1 minute at 4000 rpm (Universal 32) and supernatant discarded. Cells were then resuspended in 50µl 7-AAD (5µg/ml; Sigma). Cells were acquired immediately using three colour flow cytometry using a Becton Dickinson FACSCalibur flow cytometer. A minimum of 5000 gated events were collected in list mode format and analysis performed using CellQuest software.

T lymphocytes

After incubation overnight with desired stimulus, as described earlier, 100µl of EDTA and 20µl Intragam was added to each tube, vortexed and incubated for 15 minutes at room temperature (**Figure 2.19**). During this time, 3µl of anti-CD3 PE (Dako Corporation, Carpinteria, CA, USA) was added to each flow cytometry tube. After vortexing the blood

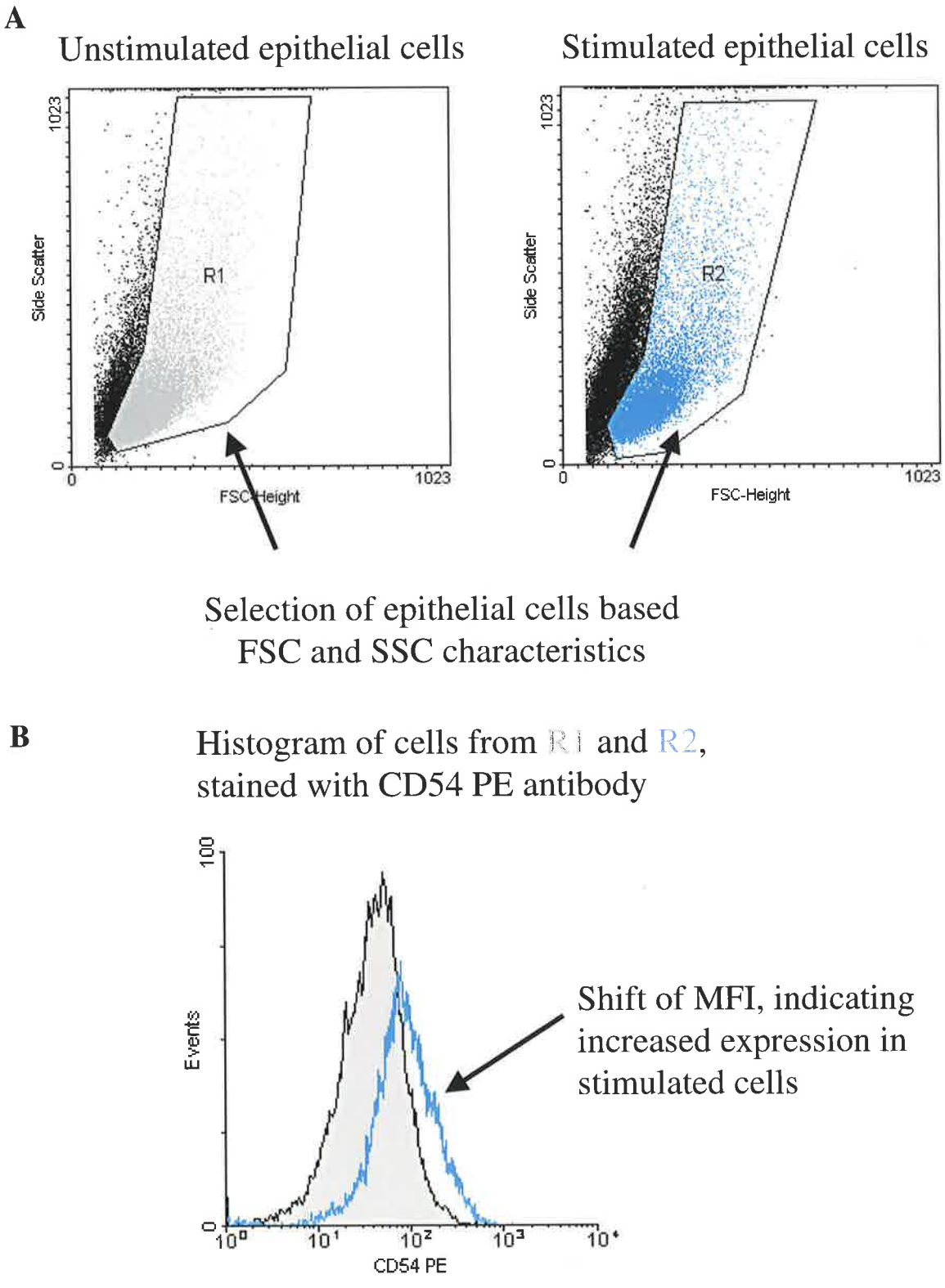


Figure 2.18: Example of flow cytometric analysis of surface marker expression by airway epithelial cells. **A**) Dot plots showing selection of airway epithelial cells from unstimulated or stimulated cell treatments, based on FSC and SSC characteristics. **B**) Histogram of epithelial cells, selected from dot plots in (**A**), showing mean fluorescent intensity (MFI) of CD54 surface marker expression. Note the shift of the MFI curve to the right demonstrating increased expression by stimulated cells.

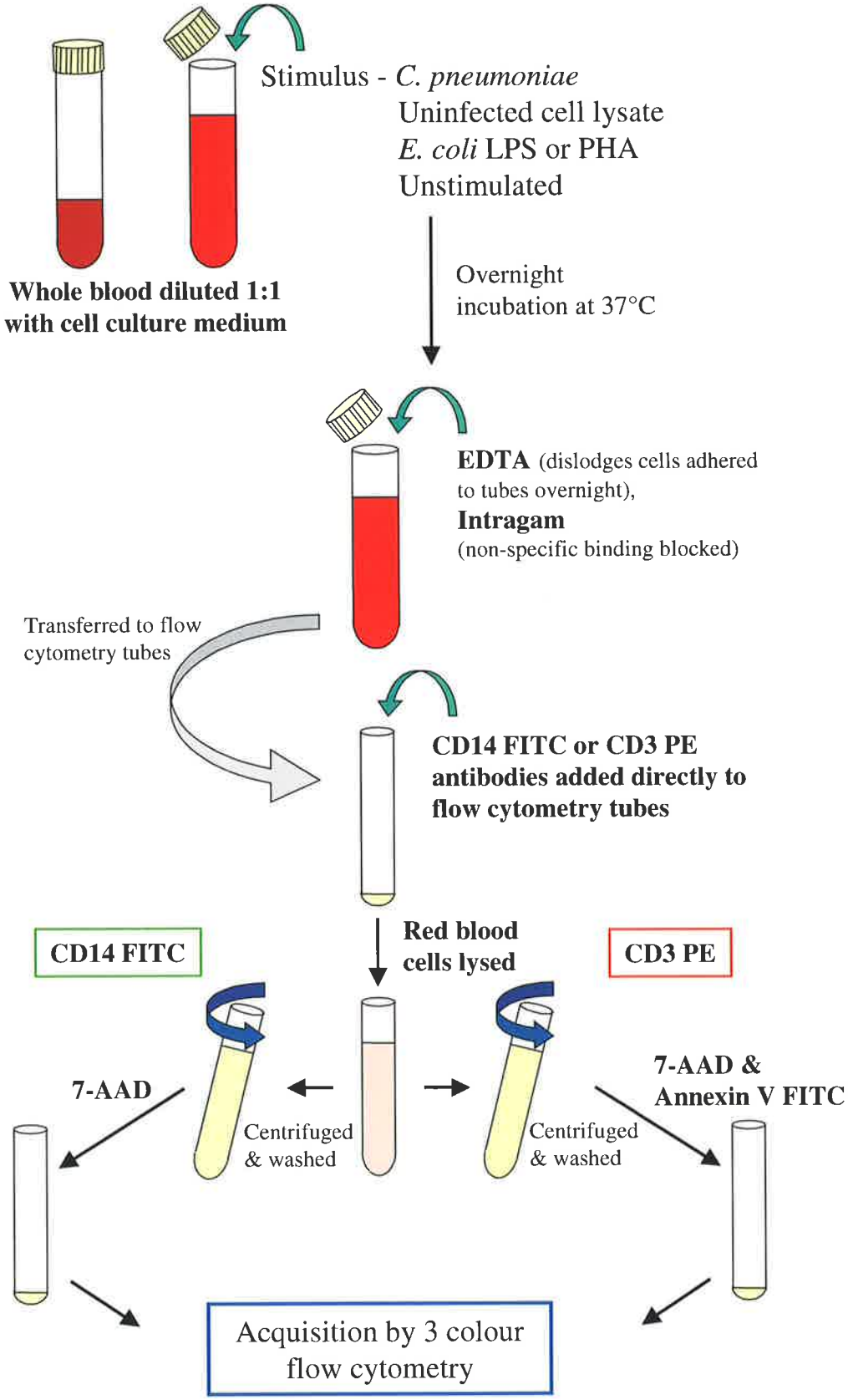


Figure 2.19: Preparation of whole blood for measurement of cell viability by flow cytometry.
Whole blood was collected into tubes containing sodium heparin as an anticoagulant and blood diluted 1:1 with cell culture media. Blood was then stimulated overnight with the desired stimulus at 37°C. The following day cells which may have adhered to tubes overnight were dislodged with EDTA (20mM) before transferring to flow cytometry tubes already containing anti-CD14 FITC or anti-CD3 PE for detection of monocytes or lymphocytes respectively. Red blood cells were lysed with Red Blood Cell Lysis buffer and cells were then washed with wash buffer or Annexin V binding buffer. To determine monocyte viability cells were resuspended in 7-AAD and acquired immediately. Lymphocyte viability was determined by incubating the cells with Annexin V FITC and resuspended in 7-AAD before immediate acquisition using by 3 colour flow cytometry using a Becton Dickinson FACSCalibur flow cytometer.

briefly, 200µl of blood was transferred to each flow tube, already containing anti-CD3 PE and incubated for a further 10 minutes at room temperature in the dark. Red blood cells were lysed by adding 2ml Red Blood Cell Lysis Buffer (Sigma) and incubating for a further 10 minutes at room temperature. Tubes were then centrifuged for 1 minute at 4000rpm (Universal 32) and supernatant decanted. Cells were then washed in 2ml annexin binding buffer (3ml HEPES (10mM HEPES/NaOH, pH 7.4), 150mM NaCl, 5mM $CaCl_2$, 1mM $MgCl_2$, 1.8mM $CaCl_2$) before centrifuging for 1 minute at 4000rpm (Universal 32) and supernatant discarded. Apoptotic and necrotic cells were determined by adding 2µl Annexin V FITC (BD Pharmingen, San Diego, CA) and resuspended in 50µl 7-AAD (5µg/ml; Sigma) before immediately acquiring. Three colour flow cytometry was performed using a Becton Dickinson FACSCalibur flow cytometer. A minimum of 10000 gated events were collected in list mode format and analysis performed using CellQuest software.

Airway epithelial cells

Airway epithelial cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate or 20ng/ml TNF-α (Promega Corporation) in 10ml polystyrene tubes at a concentration of $1x10^6$ cells/ml in a total volume of 2ml (**Figure 2.20**). The following day cells were gently resuspended using a tuberculin syringe (Terumo Medical Corporation, Elkton, MD) and 200µl transferred into flow cytometry tubes before centrifuging for 1 minute at 4000rpm (Universal 32) to remove supernatant. Cells were then washed in 2ml annexin binding buffer before centrifuging for 1 minute at 4000rpm (Universal 32) and supernatant discarded. Apoptotic and necrotic cells were determined by adding 2µl Annexin V FITC (BD Pharmingen) and resuspended in 50µl Propidium Iodide (5µg/ml; Sigma) before immediate acquisition of cells using a Becton Dickinson FACSCalibur flow cytometer. A minimum of 10000 gated events were collected in list mode format and analysis performed using CellQuest software.

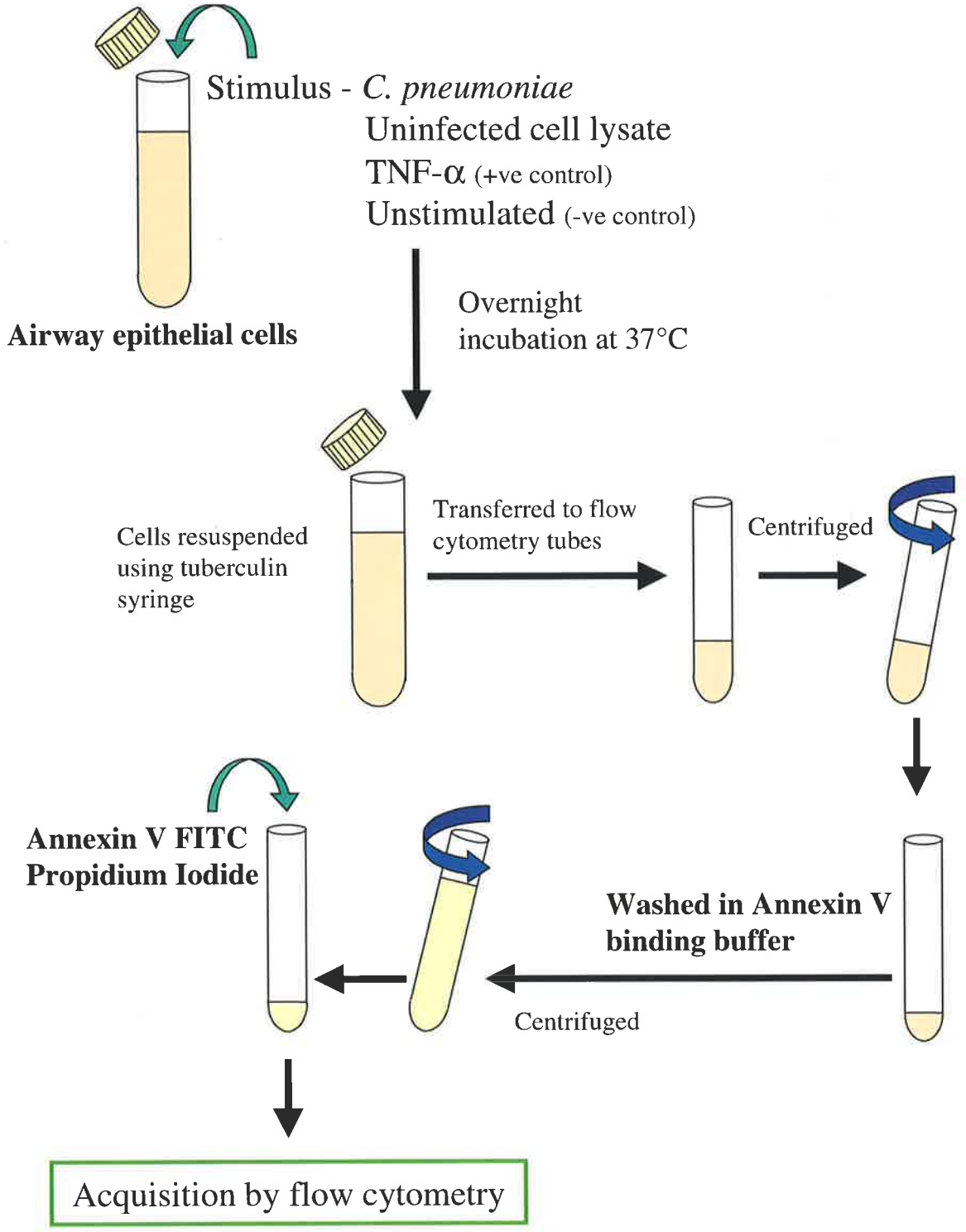


Figure 2.20: Preparation of airway epithelial cells for measurement of cell viability by flow cytometry. Airway epithelial cells were incubated overnight with the desired stimulus at 37°C. The following day, cells were resuspended using a tuberculin syringe and cells transferred to flow cytometry tubes. Tubes were centrifuged and supernatant discarded before washing in Annexin V binding buffer. Cells were then incubated with Annexin V FITC and resuspended in Propidium Iodide before immediate acquisition using a Becton Dickinson FACSCalibur flow cytometer.

Detection of cytokines by Enzyme Linked Immunosorbent Assay (ELISA)

Interleukin-8

IL-8 protein released into airway epithelial cell supernatant was also measured using an Enzyme Linked Immunosorbent Assay (ELISA) previously developed within our laboratory. Nunc Maxisorp 96 well plates were sensitised with 100µl recombinant human IL-8, 15ng/ml (Amgen, Thousand Oaks, CA, USA) diluted 0.01M PBS, pH 7.4 at room temperature for 4 hours. Wells were subsequently washed 3 times with 400µl per well with wash buffer (0.01M PBS, 0.05% Tween 20). Non-specific binding sites were blocked with 200µl blocking buffer (0.01M PBS, 1% horse serum, 0.05% sodium azide). Plates were incubated for a minimum of 30 minutes at room temperature. Wells were once again washed 3 times with wash buffer before adding 50µl primary antibody diluent (0.1M PBS, 0.05% Tween 20, 1% horse serum, 0.05% sodium azide), to wells where standard curve would be loaded and to negative control wells. The standard curve was prepared and loaded by adding 50µl of 100ng/ml of human recombinant IL-8 to the top row of wells of the standard curve and 1:1 serial dilutions were performed down the column of wells. Samples to be assayed for IL-8 were loaded into wells as neat solutions. All samples and control wells were loaded in duplicate. The primary antibody, goat anti-human IL-8 neutralising antibody (R&D Systems, Minneapolis, MN USA) was diluted 1/2000 in primary antibody diluent and 50µl added to each well (except the negative control for the primary antibody to which an additional 50µl of primary antibody diluent was added) and incubated overnight at 4°C. The following day, plates were washed 3 times with wash buffer before 100µl of secondary antibody, biotinylated rabbit anti-goat Immunoglubulins (Dako Corporation) diluted 1/40000 in secondary antibody diluent (0.1M PBS, 0.05% Tween 20, 1% Horse serum) was added to each well and incubated for 1 hour at 37°C. Wells were washed 3 times with wash buffer and 100µl of tertiary antibody, Immunopure® streptavidin, Horseradish peroxidase conjugated (Pierce, Rockford, IL, USA) diluted 1/2000 in secondary antibody diluent and incubated for 1 hour at 37°C. Wells were then washed 4 times with wash buffer and 100µl of substrate solution (20ml citrate phosphate

buffer, pH 5, 10mg *o*-phenylenediamine dihydrochloride tablet, 7µl 30% hydrogen peroxidase, added just before use) added to each well and incubated for 30 minutes at room temperature in the dark. Colour development was stopped by the addition of 100µl per well of 2.5M sulphuric acid. The plates were then read at an optical density of 490nm with 620nm as a reference wavelength on a Dynateck ELISA reader (MR7000 EIA-CALC, Dynateck, Chantilly, VA, USA).

Statistical Analysis

Two main types of parametric statistical tests were used to analyse the data generated in the current body of work. Data generated using immortalised airway epithelial cell lines were analysed with an analysis of variance (ANOVA) and were adjusted for inter-experimental variability (day and time). Data generated from whole blood samples from different subjects were analysed using a mixed model ANOVA. The mixed model ANOVA took account of multiple measures per person (triplicate data sets). All post-hoc analyses were performed using t-tests to compare least squares means estimated from the model. Data that was not normally distributed was first transformed (as indicated in figure legends) to normality before applying parametric tests for analysis. In these cases, graphical representation of the data is displayed in box plots, reporting the median and interquartile ranges. All remaining graphical data is displayed in bar graphs representing the mean and standard error of the mean unless otherwise stated. Significant differences were indicated when P values were ≤ 0.05. In all cases, P values were not adjusted for multiple comparisons as we had determined a priori hypotheses.

All statistical analyses were performed by experienced statisticians within the Department of Public Health, University of Adelaide, Adelaide, SA, Australia.

CHAPTER 3

RESULTS

Cytokine response and surface marker profiles of airway epithelial cells stimulated with *C. pneumoniae*

Introduction

The airway epithelium serves as the first line of defence for the respiratory system and has previously been thought to primarily function as a physical barrier to external stimuli and potential pathogens. However, it is now known that airway epithelium actively participates in immune responses. These cells are capable of responding to potential pathogens and playing a role in the initiation and regulation of the immune response via synthesis and production of cytokines and other immune mediators. The expression of cytokines by airway epithelial cells provides a platform for interactions with inflammatory cells important in orchestrating an effective immune response to stimuli and potential pathogens.

Airway epithelial cells express a number of cytokines including IL-8, IL-6, TNF-α, IL-1β and GM-CSF, the expression of which are increased by bronchial epithelium from asthmatics compared to control patients (137,163-166). In COPD patients, IL-8 expression by bronchial epithelial cells is also significantly increased compared to healthy subjects (167,168). These cytokines play an important role in inflammatory processes and participate in recruitment and activation of inflammatory cells within the respiratory system to combat potential pathogens such as *C. pneumoniae*.

Airway epithelial cells also express a number of molecules on the surface of their cell membrane and play a role in regulating the interaction between epithelial cells and inflammatory cells. Airway epithelial cells constitutively express markers that are involved in

adhesion including ICAM-1, ICAM-2, CD58 (LFA-3) (185,188,197). It is also suggested that vascular cell adhesion molecule-1 (VCAM-1) is induced and ICAM-1 expression is modulated by cytokines such as TNF-α, IL-1β, IFN-γ and IL-4 (155,188,197). Bacterial and viral infection of bronchial epithelium are also shown to increase the expression of ICAM-1 (148,189,190).

Although airway epithelial cells are not typically classified as professional antigen presenting cells they do play a role in this process and constitutively express MHC Class I and Class II molecules (188,190,193). Both molecules are shown to be modulated by inflammatory mediators and infection with viruses (190,201,211).

After adhesion between antigen presenting cells and T cells, and antigen presentation by accessory cells, effective immune responses by T lymphocytes also require costimulation usually provided through expression of surface molecules including CD80, CD86, CD40 and newly described markers such as B7-H2. Although there is conflicting evidence of CD80 and CD86 expression by airway epithelial cells (203,206), there are other publications documenting the expression of other costimulatory molecules CD40 and the newly described B7-H2 molecule on airway epithelium (188,207,211).

There are relatively few reports investigating surface molecule expression by airway epithelium in chronic airways disease. However, studies have shown an increase in ICAM-1 and MHC Class II molecule expression by bronchial epithelial cells of asthmatics and chronic bronchitis patients in comparison to those obtained from healthy control subjects (191,193).

There are several publications demonstrating a relationship between chronic airways diseases such as asthma and COPD, and *C. pneumoniae* infection (83,99,105). There are however, very few papers specifically addressing the relationship between *C. pneumoniae* and airway

epithelium. *C. pneumoniae* is suggested to increase IL-8 expression by both primary bronchial epithelium as well as bronchial epithelial cell lines (127,183). A limited number of studies have investigated the expression of other cytokines including IL-6, TNF-α, IL-1β and IFN-γ by *C. pneumoniae* infected airway epithelial cell lines but have failed to detect cytokine protein expression or did not observe any modulation by the bacteria (183,184). The knowledge of modulation of airway epithelial surface marker expression by *C. pneumoniae* has also been poorly investigated. Studies have been limited to bronchial epithelial cell line expression of ICAM-1 after infection with *C. pneumoniae* and show a significant upregulation (127). The expression of other molecules involved in adhesion, antigen presentation and costimulation have not been addressed.

Therefore, research to date suggests that *C. pneumoniae* may contribute to inflammation associated with chronic lung disease by modulating the cytokine expression profile of airway epithelium. However, it has not been clearly determined which cytokines are involved in this process. Further, chronic infection of the airways with *C. pneumoniae* has been documented and suggests that the response of the immune system is inadequate or compromised and allows development of persistent infection. This phenomenon may indicate problems with the recruitment and activation of additional inflammatory cells, such as neutrophils and monocytes, which are important steps in lung defence. Key mechanisms such as the expression of surface molecules, governing the interaction between airway epithelial cells and inflammatory cells may also be compromised by *C. pneumoniae* infection. It is therefore important to better understand the immune response of airway epithelial cells to *C. pneumoniae*. This knowledge may lead to a clearer understanding of how the airway epithelium contributes to the pattern of inflammation associated with chronic lung disease.

Aims

The overall aim of the studies presented in this chapter was to investigate the host-pathogen relationship by describing the cytokine and surface marker expression profile of airway epithelial cells after exposure to *C. pneumoniae*.

The specific aims of these studies were:

a) To demonstrate that *C. pneumoniae* stimulates airway epithelial cells to produce IL-8

b) To determine if different *C. pneumoniae* isolates modulate IL-8 expression in bronchial epithelium in a similar manner

c) To investigate whether bronchial epithelial cell expression of cytokines other than IL-8 are modulated by *C. pneumoniae*

d) To investigate whether *C. pneumoniae* modulates airway epithelial cell surface marker expression

Methods

Immortalised airway epithelial cell lines were used to investigate the host-pathogen relationship between airway epithelium and *C. pneumoniae*. Two bronchial epithelial cell lines, 16HBE14o- and BEAS-2B were used in these studies and were maintained as described in Chapter 2. *C. pneumoniae* was propagated and harvested in HEp-2 cells as described in Chapter 2. To assess the interaction between airway epithelium and *C. pneumoniae* we measured the immune response in terms of cytokine production. Cells were seeded at $4x10^5$ cells/ml (16HBE14o-) or $3x10^5$ cells/ml (BEAS-2B) in the appropriate base media in 24 well tissue culture plates. The cells were incubated overnight, allowing adherence to tissue culture plates and cell monolayers to reach approximately 80% confluence prior to stimulation. Cell media was then replaced with fresh media containing the desired stimulus. In a final volume of 1ml, cells were stimulated with live *C. pneumoniae* (previously titrated in HEp-2 cells as described in Chapter 2), a comparable concentration of uninfected cell lysate (which served as a negative control), or TNF-α (20ng/ml; which served as a positive control) or cells were incubated in media alone which served as an unstimulated control.

We investigated the response of airway epithelial cells to *C. pneumoniae* stimulation by measuring the release of cytokines into cell culture supernatant. Cell culture supernatant was collected after 24 hours of stimulation and stored at -70°C until assayed. Initially we measured IL-8 release into cell culture supernatant by an ELISA developed within our laboratory and described in Chapter 2. Subsequently, a wider range of cytokines including IL-8, IL-1β, TNF-α, IL-6, IL-10 and IL-12p70 released into cell culture supernatant were measured by flow cytometry using a Human Inflammation Cytometric Bead Array kit. This kit utilises a bead capture assay that has the advantage of measuring up to six soluble proteins simultaneously. A detailed description of this assay can be found in Chapter 2.

The response of airway epithelial cells to *C. pneumoniae* was also investigated by measuring the expression of cell surface molecules. In a final volume of 1ml, BEAS-2B cells were resuspended at a density of $3x10^5$ cells/ml and were incubated overnight in 10ml polystyrene tubes, placed at an angle, with the desired stimulus. Cells were stimulated with *C. pneumoniae*, a comparable concentration of uninfected cell lysate (which served as a negative control), or TNF-α (20ng/ml; which served as a positive control) or incubated in media alone which served as an unstimulated control. The following day cells were processed to measure the expression of surface molecules important in immune responses by flow cytometry as described in Chapter 2.

Results

Does C. pneumoniae stimulate airway epithelial cells to produce IL-8?

We initially measured the response of airway epithelial cells to *C. pneumoniae* stimulation in terms of IL-8 expression. After overnight stimulation with TNF-α, BEAS-2B cells significantly increased IL-8 release into cell culture supernatant compared to unstimulated cells, as measured by ELISA (**Figure 3.1**). This confirmed that BEAS-2B cells could be stimulated to increase their expression of IL-8. Also shown in **Figure 3.1**, we demonstrated that *C. pneumoniae* (IOL-207) increased IL-8 expression by BEAS-2B cells, compared to comparable amounts of uninfected cell lysate. In these experiments, there was no significant difference between unstimulated cells and those cells incubated with uninfected cell lysate which then made it possible to compare different concentrations of *C. pneumoniae*. We found that increasing concentrations of *C. pneumoniae* caused a significantly increased concentration of IL-8 to be released into cell culture supernatant (**Figure 3.1**).

After confirming that IL-8 was released into BEAS-2B cell culture supernatant after stimulation with *C. pneumoniae*, we next investigated whether an alternative bronchial epithelial cell line, 16HBE14o-, responded in a similar manner. Although we were able to consistently detect IL-8 released from 16HBE14o- cells under basal conditions, we found that the concentration of IL-8 released was not significantly changed after stimulation with *C. pneumoniae* at any of the concentrations tested (**Figure 3.2**). In light of these results, all future experiments investigating the response of airway epithelium to stimulation with *C. pneumoniae* were performed using the BEAS-2B cell line.

Do different C. pneumoniae isolates increase IL-8 production in bronchial epithelium?

To determine whether the increase in IL-8 by airway epithelium was specific to the IOL-207 *C. pneumoniae* isolate, we evaluated a second *C. pneumoniae* isolate, WA97001 (386). Similar to the experiments performed with the original isolate (IOL-207), we stimulated BEAS-2B cells with increased concentrations of *C. pneumoniae* and measured IL-8 release

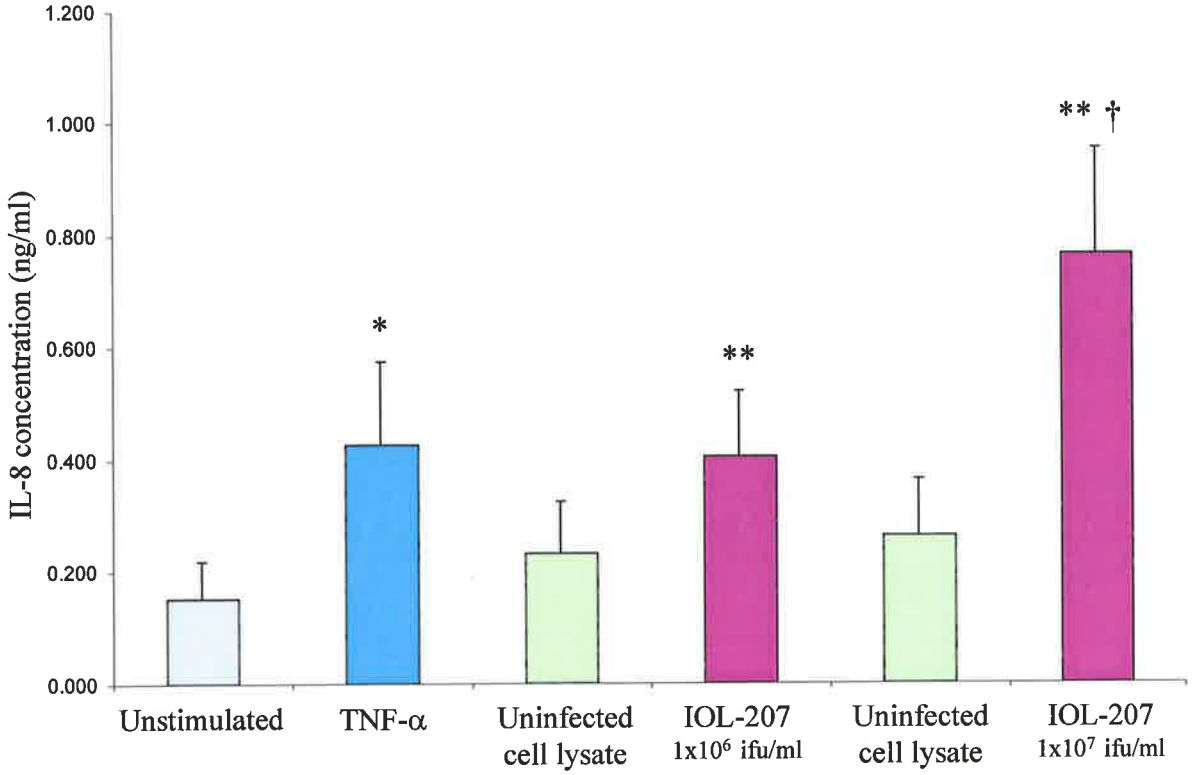


Figure 3.1: *C. pneumoniae* (IOL-207) increases IL-8 protein expression by BEAS-2B cells.
BEAS-2B cells were plated into 24 well tissue culture flasks at a density of $3x10^5$ cells/ml and allowed to adhere overnight. Cells were then stimulated for 24hrs with TNF-α (20ng/ml), uninfected cell lysate (volume equivalent to *C. pneumoniae* preparations) or *C. pneumoniae* (IOL-207). Cell supernatant was collected and assayed for IL-8 content by ELISA. Bars represent mean ± SEM of 9 separate experiments, performed in triplicate. * $p< 0.05$ compared to unstimulated cells, regression analysis. ** $p<0.05$ compared to comparable amount uninfected cell lysate, ANOVA. † $p<0.05$ compared to IOL-207, $1x10^6$ ifu/ml, ANOVA.

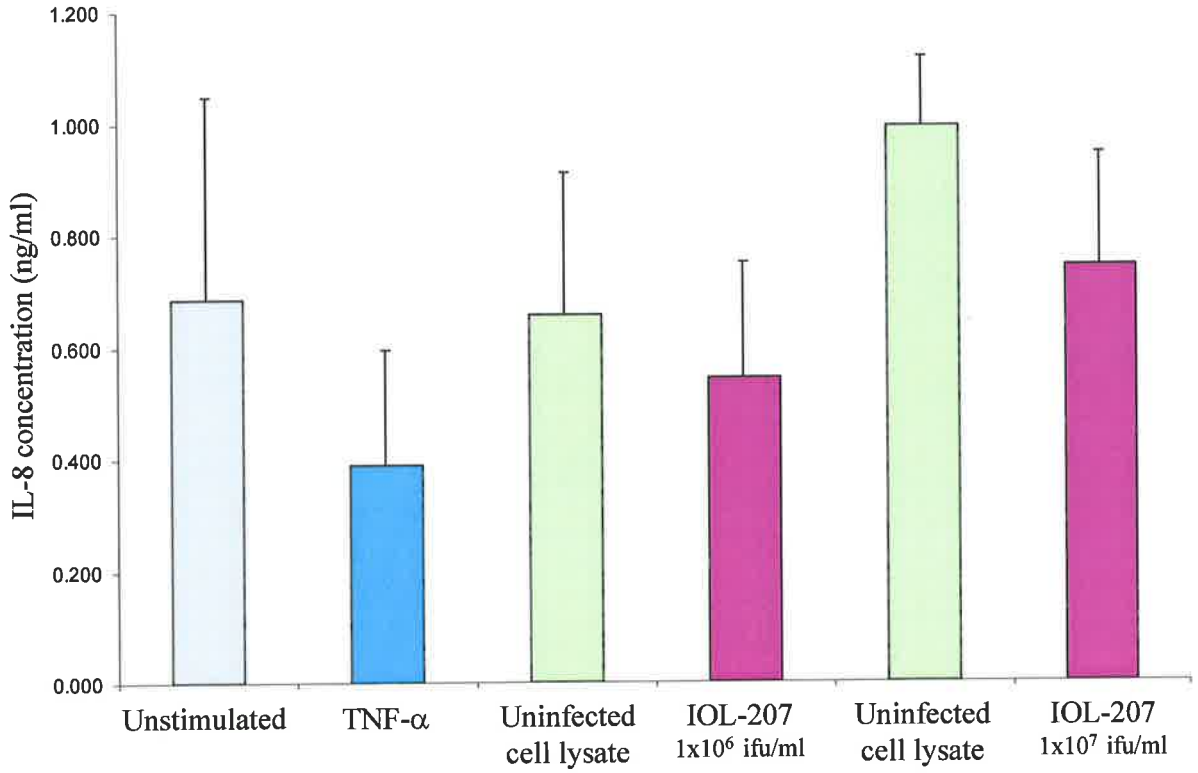


Figure 3.2: *C. pneumoniae* (IOL-207) has no effect on IL-8 protein expression by 16HBE14o- cells. 16HBE cells were plated into 24 well tissue culture flasks at a density of $4x10^5$ cells/ml and allowed to adhere overnight. Cells were then stimulated for 24hrs with TNF-α (20ng/ml), uninfected cell lysate (volume equivalent to *C. pneumoniae* preparations) or *C. pneumoniae* (IOL-207). Cell supernatant was collected and assayed for IL-8 content by ELISA. Bars represent mean ± SEM of 3 separate experiments, performed in triplicate.

into cell culture supernatant by ELISA. Consistent with the results obtained with the IOL-207 *C. pneumoniae* isolate, WA97001 significantly increased the concentration of IL-8 released into the supernatant of BEAS-2B cells compared to cells stimulated with uninfected cell lysate (**Figure 3.3**). BEAS-2B cells stimulated with $1x10^6$ ifu/ml of WA97001 did not cause any further increase in IL-8 concentration in BEAS-2B cell supernatant than that measured when cells were stimulated with $1x10^5$ ifu/ml of the WA97001 *C. pneumoniae* isolate (**Figure 3.3**) suggesting that production was maximised at the lower dose. The concentration of IL-8 detected in BEAS-2B cell supernatant was up to 3.5 fold higher after stimulation with WA97001 in comparison to an equivalent concentration of the IOL-207 isolate (see **Figure 3.1**), suggesting that the WA97001 isolate may be more potent at eliciting this response.

Does C. pneumoniae stimulate cytokine production by bronchial epithelial cells other than IL-8?

After confirming that IL-8 release into BEAS-2B cell culture supernatant was significantly increased by two different isolates of *C. pneumoniae*, we next investigated whether the release of other cytokines into cell culture supernatant was also altered. Cell cultures were set up using BEAS-2B cells and stimulated overnight with *C. pneumoniae*, as described for the first aim of this chapter. Cell culture supernatant was then analysed for the presence of cytokines using the Human Inflammation Cytometric Bead Array kit. This kit allowed the simultaneous detection of IL-8 as well as other cytokines including IL-1β, TNF-α, IL-6, IL-10 and IL-12p70measured by flow cytometric techniques.

As shown in **Figure 3.4**, measurement of IL-8 using the Cytometric Bead Array kit, confirmed the results obtained using the ELISA technique. Consistent with the data obtained for the ELISA, *C. pneumoniae* increased IL-8 release into BEAS-2B cell supernatant compared to uninfected cell lysate. When cells were stimulated with the IOL-207 isolate, IL-8 release was only increased when the highest concentration ($1x10^7$ ifu/ml) of *C. pneumoniae* was used. Whereas, BEAS-2B cells incubated overnight with the WA97001

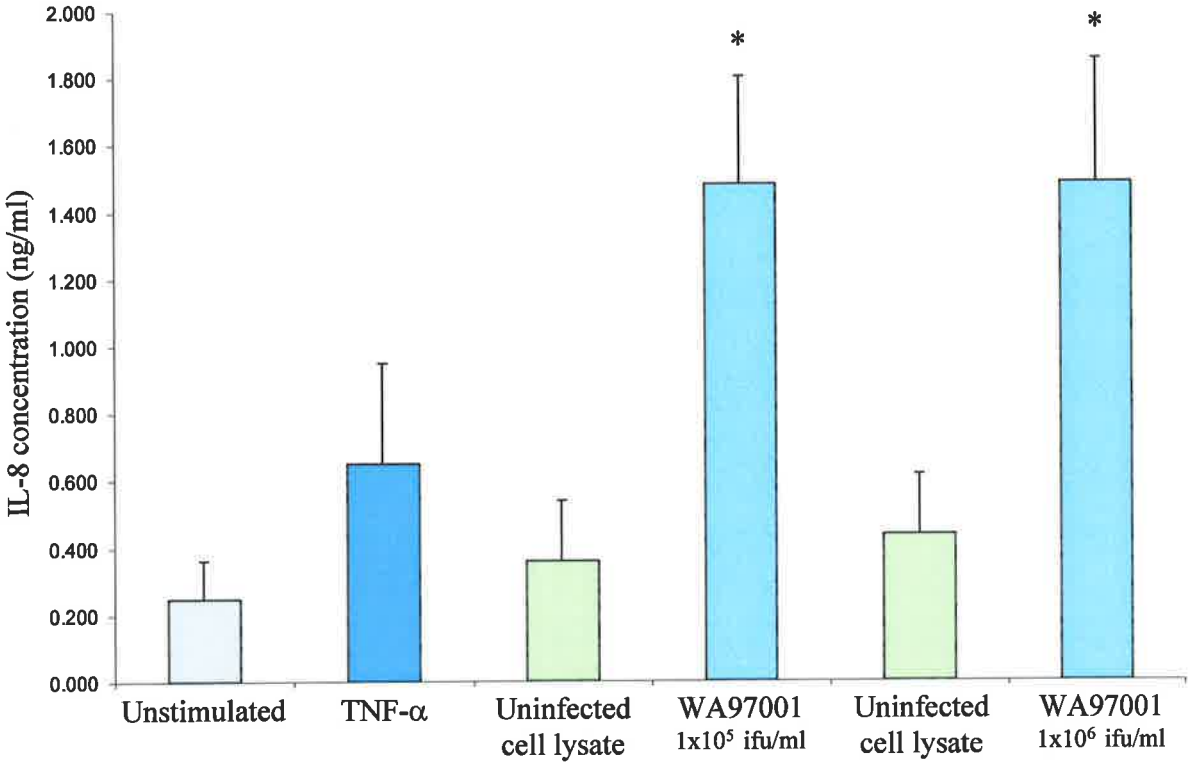


Figure 3.3: *C. pneumoniae* (WA97001) increases IL-8 protein expression by BEAS-2B cells.
BEAS-2B cells were plated into 24 well tissue culture flasks at a density of $3x10^5$ cells/ml and allowed to adhere overnight. Cells were then stimulated for 24hrs with TNF-α (20ng/ml), uninfected cell lysate (volume equivalent to *C. pneumoniae* preparations) or *C. pneumoniae* (WA97001). Cell supernatant was collected and assayed for IL-8 content by ELISA. Bars represent mean ± SEM of 4 separate experiments, performed in triplicate. * $p<0.05$ compared to comparable amount uninfected cell lysate, regression analysis.

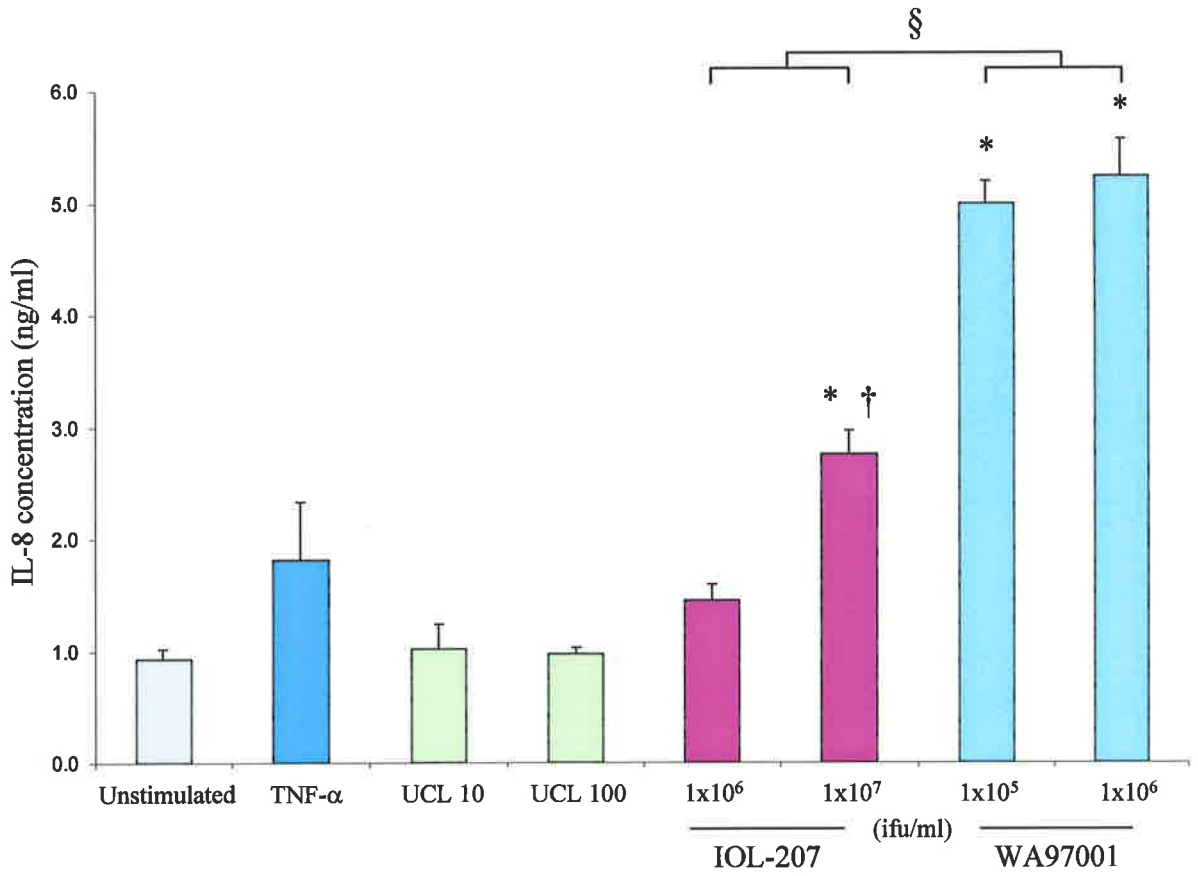


Figure 3.4: *C. pneumoniae* increases IL-8 protein expression by BEAS-2B cells.
BEAS-2B cells were plated into 24 well tissue culture flasks at a density of $3x10^5$ cells/ml and allowed to adhere overnight. Cells were then stimulated for 24hrs with TNF-α (20ng/ml), uninfected cell lysate (volume equivalent to *C. pneumoniae* preparations) or *C. pneumoniae* (IOL-207 or WA97001). Cell supernatant was collected and assayed for IL-8 content by flow cytometry using a Cytometric Bead Array kit. Bars represent the mean ± SEM of one of three experiments, performed in triplicate. * $p<0.05$ compared to comparable amount uninfected cell lysate, ANOVA. † $p<0.05$ compared to IOL-207, $1x10^6$ ifu/ml, ANOVA. § $p<0.05$, WA97001 versus IOL-207, ANOVA.

isolate, increased IL-8 production at a concentration as low as $1x10^5$ ifu/ml was used. The level of IL-8 induction by WA97001 was not increased further when the concentration was increased to $1x10^6$ ifu/ml but was maintained at 5ng/ml. IL-8 protein detected in cell culture by the Cytometric Bead Array kit yielded similar concentrations to those measured using the ELISA method and showed a 3.5 fold increase in IL-8 induced by WA97001 in comparison to an equivalent concentration of IOL-207.

The Cytometric Bead Array kit also allowed the measurement of IL-6 in cell supernatant. As was observed for IL-8, both isolates of *C. pneumoniae* (IOL-207 and WA97001) stimulated BEAS-2B cells to release IL-6 into cell supernatant (**Figure 3.5**). Also consistent with the results obtained for IL-8, IL-6 release by BEAS-2B cells was only induced by the IOL-207 isolate at the highest concentration used ($1x10^7$ ifu/ml). IL-6 expression was also significantly increased after stimulation with the WA97001 isolate at all concentrations used. The level of IL-6 release into cell supernatant induced after stimulation with the WA97001 isolate was greater than that measured after stimulation with the IOL-207 isolate.

C. pneumoniae failed to stimulate BEAS-2B cells to induce TNF-α expression. TNF-α could only be detected in supernatant of cells which had been stimulated with TNF-α as a positive control (**Table 3.1**).

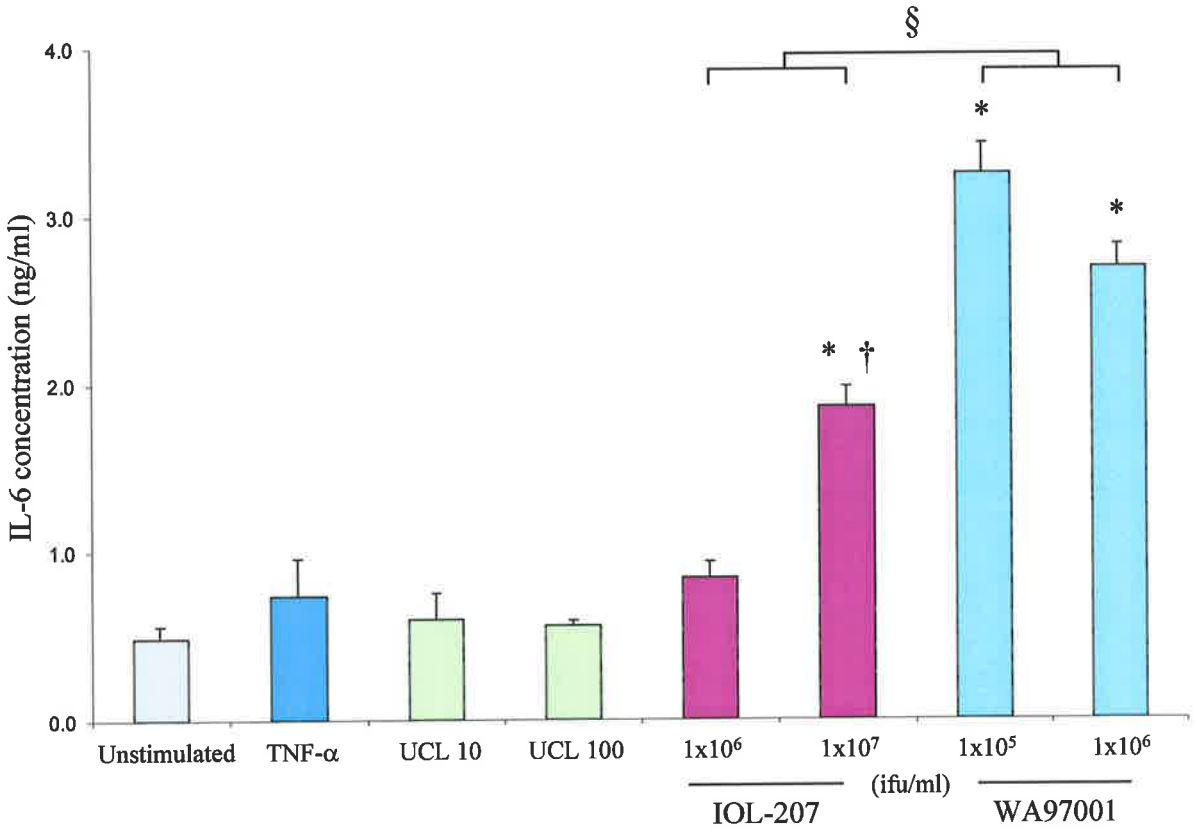


Figure 3.5: *C. pneumoniae* increases IL-6 protein expression by BEAS-2B cells.
BEAS-2B cells were plated into 24 well tissue culture flasks at a density of $3x10^5$ cells/ml and allowed to adhere overnight. Cells were then stimulated for 24hrs with TNF-α (20ng/ml), uninfected cell lysate (volume equivalent to *C. pneumoniae* preparations) or *C. pneumoniae* (IOL-207 or WA97001). Cell supernatant was collected and assayed for IL-6 content by flow cytometry using a Cytometric Bead Array kit. Bars represent the mean ± SEM of one of three experiments, performed in triplicate. * $p<0.05$ compared to comparable amount uninfected cell lysate, ANOVA. † $p<0.05$ compared to IOL-207, $1x10^6$ ifu/ml, ANOVA. § $p<0.05$, WA97001 versus IOL-207, ANOVA.

TNF-α (pg/ml) secretion by BEAS-2B cells after incubation with *C. pneumoniae*								
	Unstimulated	**TNF-α (20ng/ml)**	**UCL 10**	**UCL 100**	**IOL-207 (ifu/ml)**		**WA97001 (ifu/ml)**	
					$1x10^6$	$1x10^7$	$1x10^5$	$1x10^6$
	0.4	67.9	1.3	1.5	3.0	2.0	2.4	2.3
	1.3	165.9	0.7	1.1	2.4	0.6	2.6	0.5
	1.8	36.7	1.1	1.0	1.2	1.1	2.5	2.7
Mean	1.2	90.2	1.0	1.2	2.2	1.2	2.5	1.8
SEM	0.4	38.9	0.2	0.2	0.5	0.4	0.1	0.7

Table 3.1: *C. pneumoniae* does not induce TNF-α secretion by BEAS-2B cells.
BEAS-2B cells were plated at $3x10^5$ cells/ml and incubated overnight with *C. pneumoniae*. Cell culture supernatant was collected and analysed for TNF-α using a Cytometric Bead Array kit. Data represents the mean concentrations of 3 separate experiments performed in triplicate.

We also investigated the expression of IL-1β, IL-10 and IL-12 by BEAS-2B cells in response to *C. pneumoniae*. Using the Cytometric Bead Array kit, none of these cytokines were detected in unstimulated BEAS-2B cell supernatant. We also observed that BEAS-2B cells were not stimulated by TNF-α or *C. pneumoniae* to induce the expression of these cytokines.

Does C. pneumoniae modulate airway epithelial cell surface marker expression?
After confirming that *C. pneumoniae* increased cytokine expression by airway epithelial cells, our next question was to determine whether *C. pneumoniae* also altered cell surface molecule expression. Airway epithelial cells are not typically classified as antigen presenting cells but are known to express major histocompatibility complex molecules. We showed that the majority of unstimulated BEAS-2B cells expressed major histocompatibility class I (HLA-ABC) molecules (**Table 3.2**). The percentage of cells expressing these molecules after stimulation with *C. pneumoniae* (WA97001) was unchanged compared to uninfected cell lysate. Similarly, the amount per cell, as determined by the mean fluorescent intensity, also remained unaltered after *C. pneumoniae* (WA97001) stimulation (**Figure 3.6**). Experiments were also performed using the IOL-207 *C. pneumoniae* isolate but the results were similar to those obtained with the WA97001 isolate and had no effect on the expression of MHC Class I molecules by BEAS-2B cells.

Percentage of BEAS-2B cells expressing MHC Class I molecules						
	Unstimulated	TNF-α (20ng/ml)	UCL 10	UCL 100	WA97001 (ifu/ml)	
					$1x10^5$	$1x10^6$
	91.86	89.53	87.18	86.01	93.87	94.39
	99.75	99.68	99.72	98.60	99.58	98.46
	96.13	94.46	92.87	81.70	97.02	96.57
Mean	97.94	97.07	96.30	90.15	98.30	97.51
SEM	1.81	2.61	3.42	8.45	1.28	0.94

Table 3.2: *C. pneumoniae* does not alter the percentage of BEAS-2B cells expressing MHC Class I molecules. BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with TNF-α (20ng/ml), *C. pneumoniae*, or a comparable amount of uninfected cell lysate. Cell surface expression of MHC Class I molecules were detected using an anti-human monoclonal HLA-ABC FITC conjugated antibody and measured using flow cytometry. Data represents the mean percentage of BEAS-2B cells expressing MHC Class I molecules of 3 separate experiments performed in triplicate.

We also investigated the expression of MHC Class II molecules on BEAS-2B cells. However, we were unable to detect MHC Class II molecules on unstimulated cells and it was not induced after overnight stimulation under any of the tested conditions.

The expression of the adhesion molecule ICAM-1 was also measured on BEAS-2B cells that had been stimulated with *C. pneumoniae* (WA97001). The majority of unstimulated BEAS-2B cells expressed ICAM-1 on their surface. The percentage of BEAS-2B cells expressing ICAM-1 remained on average over 70% and was not significantly changed by *C. pneumoniae* compared to uninfected cell lysate (**Table 3.3**). Similarly, the MFI of BEAS-2B cells expressing ICAM-1 was not significantly changed under any of the treatment conditions tested (**Figure 3.7**). Experiments were also performed using the IOL-207 *C. pneumoniae* isolate but the results were similar to those obtained with the WA97001 isolate and had no effect on the expression of ICAM-1 by BEAS-2B cells.

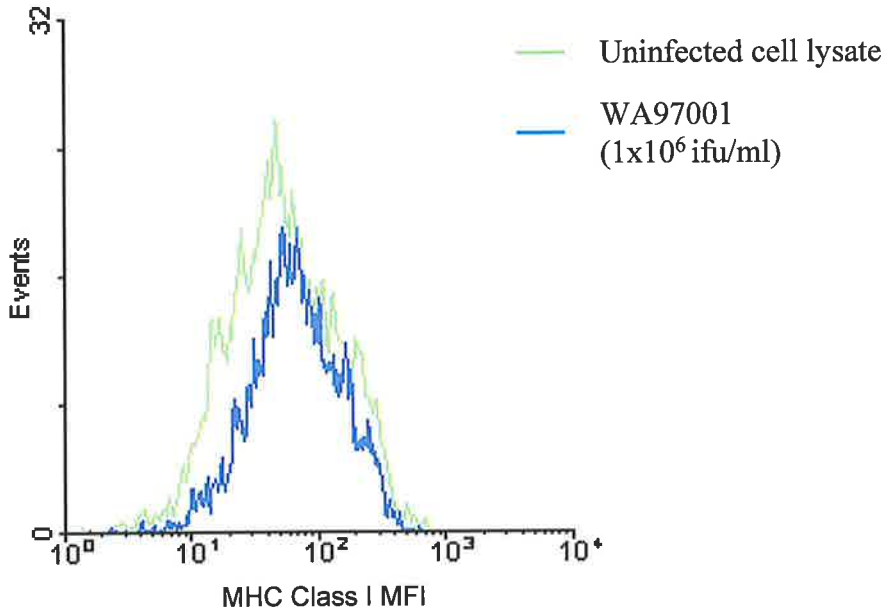


Figure 3.6: *C. pneumoniae* has no effect on BEAS-2B MHC Class I MFI.
BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with *C. pneumoniae* (WA97001; $1x10^6$ ifu/ml) or a comparable amount of uninfected cell lysate. Cell surface expression of MHC Class I molecules were detected using an anti-human monoclonal HLA-ABC FITC conjugated antibody and MFI determined using flow cytometry. Histogram is a typical representation of MHC Class I MFI by BEAS-2B cells after overnight stimulation with *C. pneumoniae* or uninfected cell lysate.

Percentage of BEAS-2B cells expressing ICAM-1						
	Unstimulated	TNF-α (20ng/ml)	UCL 10	UCL 100	WA97001 (ifu/ml)	
					$1x10^5$	$1x10^6$
	51.05	59.13	55.98	45.83	61.58	67.25
	90.02	92.97	90.53	89.97	82.54	85.93
	81.81	84.12	78.40	57.56	88.02	79.74
Mean	74.29	78.74	74.97	64.45	77.38	77.64
SEM	11.86	10.14	10.12	13.20	8.06	5.49

Table 3.3: *C. pneumoniae* does not alter the percentage of BEAS-2B cells expressing ICAM-1.
BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with TNF-α (20ng/ml), *C. pneumoniae*, or a comparable amount of uninfected cell lysate. Cell surface expression of ICAM-1 was detected using an anti-human monoclonal ICAM-1 PE conjugated antibody and measured using flow cytometry. Data represents the mean percentage of BEAS-2B cells expressing ICAM-1 of 3 separate experiments performed in triplicate.

The expression of a second adhesion molecule, CD58, was also examined in response to *C. pneumoniae*. We consistently detected CD58 expression on the surface of the BEAS-2B cell line. However, after exposure to uninfected cell lysate, the expression was significantly decreased. Stimulation with *C. pneumoniae* (WA97001) resulted in an increase in the percentage of cells expressing CD58 back to the levels of unstimulated cells (**Table 3.4**). There were no significant changes in the MFI of cells expressing CD58 (**Figure 3.8**). Experiments performed with the IOL-207 isolate showed similar results although the percentage of cells expressing CD58 after *C. pneumoniae* stimulation, failed to return to baseline levels.

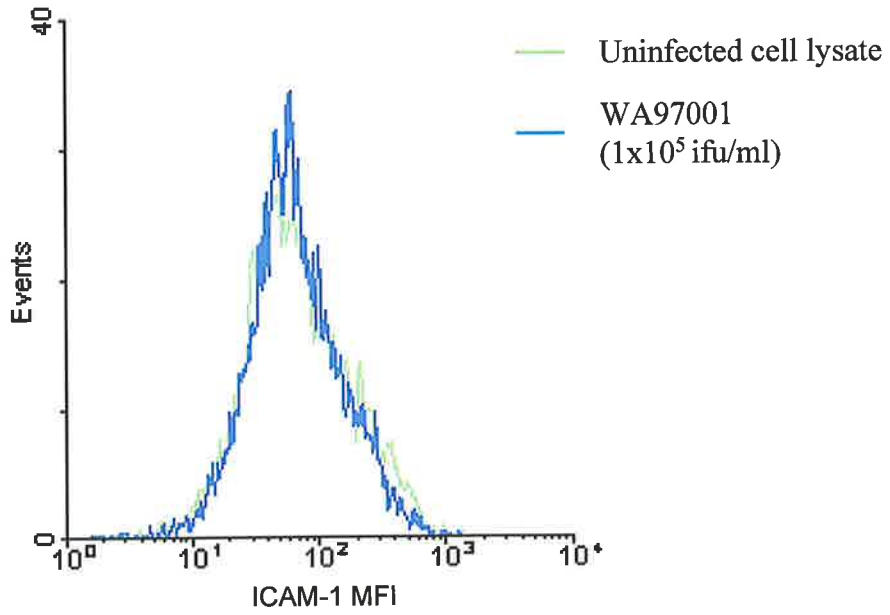


Figure 3.7: *C. pneumoniae* has no effect on MFI of BEAS-2B expressing ICAM-1.
BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with *C. pneumoniae* (WA97001; $1x10^5$ ifu/ml) or a comparable amount of uninfected cell lysate. Cell surface expression of ICAM-1 were detected using an anti-human monoclonal ICAM-1 PE conjugated antibody and MFI determined using flow cytometry. Histogram is a typical representation of ICAM-1 MFI by BEAS-2B cells after overnight stimulation with *C. pneumoniae*.

Percentage of BEAS-2B cells expressing CD58						
	Unstimulated	**TNF-α (20ng/ml)**	**UCL 10**	**UCL 100**	**WA97001 (ifu/ml)**	
					$1x10^5$	$1x10^6$
	49.47	43.74	24.51	25.50	43.77	59.65
	55.59	55.31	41.17	27.50	44.79	56.61
	52.76	48.32	36.37	23.40	51.38	55.63
Mean	52.61	49.12	34.02	25.47	46.65	57.30
SEM	1.77	3.36	4.95	1.18	2.38	1.21

Table 3.4: *C. pneumoniae* does not alter the percentage of BEAS-2B cells expressing CD58.
BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with TNF-α (20ng/ml), *C. pneumoniae*, or a comparable amount of uninfected cell lysate. Cell surface expression of CD58 was detected using an anti-human monoclonal CD58 FITC conjugated antibody and measured using flow cytometry. Data represents the mean percentage of BEAS-2B cells expressing CD58 from one experiment performed in triplicate.

We also investigated the expression of a number of other surface molecules on BEAS-2B cells including CD80, CD86, CD40, CD69 and CD25. However, we were unable to detect their expression on unstimulated cells nor were they induced after stimulation.

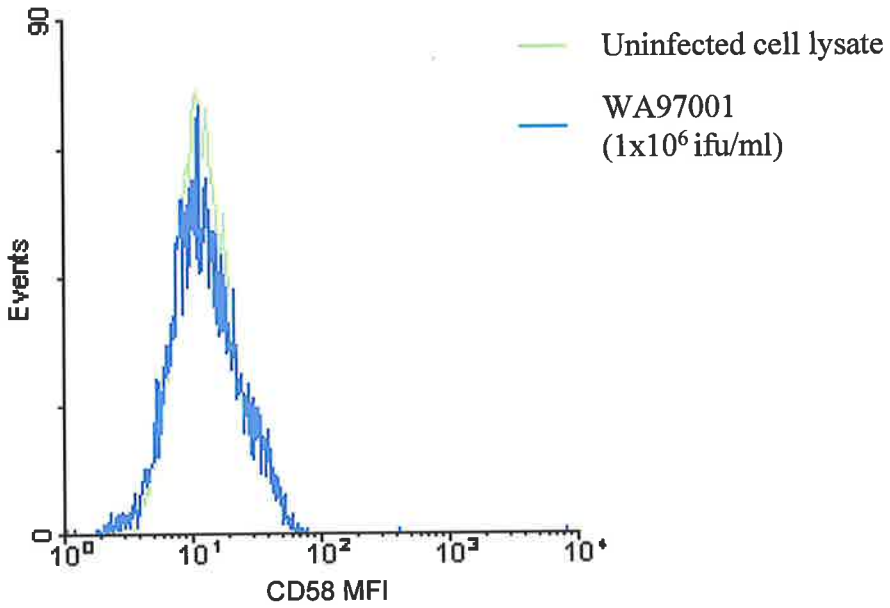


Figure 3.8: *C. pneumoniae* has no effect on MFI of BEAS-2B expressing CD58.
BEAS-2B cells were resuspended at $3x10^5$ cells/ml and incubated overnight with *C. pneumoniae* (WA97001; $1x10^5$ ifu/ml) or a comparable amount of uninfected cell lysate. Cell surface expression of CD58 were detected using an anti-human monoclonal CD58 FITC conjugated antibody and MFI determined using flow cytometry. Histogram is a typical representation of CD58 MFI by BEAS-2B cells after overnight stimulation with *C. pneumoniae*.

Discussion

The epithelium of the airways is the first line of defence against invasion of potential pathogens into the lungs. It is also ideally situated to orchestrate immune response and inflammatory processes. In the previous chapter, we showed that a variety of cells are susceptible to *C. pneumoniae* infection. In the current chapter we have investigated the response of airway epithelial cells to *C. pneumoniae* in terms of cytokine and surface marker expression.

We demonstrated that two previously untested *C. pneumoniae* isolates (IOL-207 and WA97001) increased IL-8 secretion from the BEAS-2B cell line. This is consistent with previous reports describing that airway epithelial cell lines, (BEAS-2B and A549 cells) increase the production of IL-8 in response to infection with *C. pneumoniae* (127,183). After overnight incubation with the WA97001 isolate, at $1x10^5$ ifu/ml, the levels of IL-8 measured using the Cytometric Bead Array kit, were comparable to those measured by Jahn and colleagues, using the same concentration of the *C. pneumoniae* isolate utilised in their studies (GiD) (127). Although we also measured an increase in IL-8 secretion by BEAS-2B cells using an alternative isolate, (IOL-207) a higher concentration was required to induce IL-8 and the levels were considerably less. An alternative respiratory epithelial cell line, A549, is also shown to increase IL-8 secretion after infection with *C. pneumoniae*, using a different *C. pneumoniae* isolate, CM-1 (183). A relatively high concentration of the CM-1 *C. pneumoniae* isolate ($1x10^7$ ifu/ml) was required to induce significant IL-8 production by the A549 cells, similar to our observations with the IOL-207 isolate. These results suggest that the *C. pneumoniae* isolate used to investigate the responses of airway epithelial cells may be of importance. The WA97001 isolate employed in the current studies, the GiD strain used by Jahn and colleagues and the CM-1 strain used by Yang and colleagues (although much higher concentrations were required to induce significant cytokine production) were all originally isolated from respiratory specimens (127,183,386,389). The IOL-207 isolate

however, was originally isolated from an ocular infection of trachoma (385) and similar to the CM-1 isolate (183), required increased concentrations to produce a response. Endothelial cells are also susceptible to *C. pneumoniae* infection and respond to different *C. pneumoniae* isolates with varying intensity as measured by induction of IL-8 and MCP-1 (129,390,391).

The technology of the Cytometric Bead Array kit enabled us to analyse a variety of cytokines released into cell supernatant in a time and cost efficient manner. This enabled us to also demonstrate that IL-6 secretion was increased by BEAS-2B cells stimulated with either IOL-207 or WA97001 *C. pneumoniae* isolates. The pattern of IL-6 release was similar to that of IL-8 where both isolates caused an increase in cytokine release but it appeared that the WA97001 isolate was more potent in eliciting this response. A lower concentration ($1x10^5$ ifu/ml) of WA97001 induced a larger amount of IL-6 to be secreted from the cells, as compared to IOL-207. The publication by Jahn and colleagues (127) did not include analysis of IL-6 secretion by BEAS-2B cells infected with the GiD *C. pneumoniae* isolate. In contrast to the studies by Yang and colleagues who infected A549 cells with the CM-1 *C. pneumoniae* isolate, IL-6 expression remained unaltered compared to uninfected cells (183). In a continuous infection model of *C. pneumoniae*, the prototype strain, TW-183 and CM-1, induced significant production of IL-8 and IL-6 from HEp-2 cells (a tracheal carcinoma cell line) (184). This suggests that the cytokine response to *C. pneumoniae* infection may also be dependent on the individual characteristics of the cell line used.

Adding to this suggestion, the 16HBE14o- cell line did not respond to *C. pneumoniae* in terms of IL-8 production. The responses of each cell line to *C. pneumoniae* may be a result of different conditions under which the cells are maintained. Both airway epithelial cell lines utilised in the current studies are transformed with the SV40 large T antigen (see (383) and American Type Culture Collection CRL-9069) but are maintained in different base media. The 16HBE14o- cells were maintained in a standard cell culture medium consisting of a base

media of RPMI supplemented with antibiotics and foetal calf serum. However, the BEAS-2B cells were grown in a base media of Keratinocyte-Serum Free Media, supplemented with antibiotics as well as bovine pituitary extract and epidermal growth factor. The absence of serum in the BEAS-2B cell media and addition of growth factors may be contributing factors to the difference in cellular responses to *C. pneumoniae* stimulation. In addition, the baseline levels of IL-8 production by the 16HBEo- cell line were up to 4-5 times higher than that observed with the BEAS-2B cell line. This increased level of constitutive IL-8 expression by the 16HBEo- cell line may be partly responsible for not observing any response to *C. pneumoniae*.

It is likely that the release of chemokines such as IL-8 from airway epithelial cells mediate the migration of inflammatory cells to the infection site. In a mouse model of *C. pneumoniae* infection, the early inflammatory infiltrates consist primarily of polymorphonuclear leucocytes, followed by mononuclear cells later (146). Endothelial cells and smooth muscle cells are also reported to increase IL-8 and IL-6 expression after infection with *C. pneumoniae* (24,356,392,393). In addition, infection of endothelial cells with *C. pneumoniae* causes transepithelial migration of neutrophils and monocytes and is correlated to IL-8 and MCP-1 release (356). Blocking antibodies to IL-8 or MCP-1 in these studies resulted in significant inhibition of both neutrophil and monocyte chemotaxis. *C. pneumoniae* induced IL-8 expression by BEAS-2B cells is also followed by transmigration of neutrophils (127). Although, in this study, pretreatment of epithelial cells or polymorphonuclear leucocytes with blocking antibodies to IL-8 failed to inhibit neutrophil migration.

Therefore, *C. pneumoniae* infection of the airway epithelium and subsequent IL-8 release may be involved in inflammatory cell chemotaxis. Adding to this theory are the reports documenting increased levels of IL-8 in the airways of both asthmatic and COPD patients (167,175,181,394). Although neutrophils are the predominant inflammatory cell type in

COPD as compared to asthma, it has been shown that IL-8 levels are correlated to neutrophil cell counts in both asthmatic and chronic bronchitis patients (175,181,346). In addition, IL-8 expression is shown to be increased by bronchial epithelial cells in asthmatics and COPD patients, as compared to healthy controls (164,167,168).

The predominant biological effects of IL-6 are activation, growth and differentiation of T cells and B cells (159,395). However, it is also reported that the IL-6 receptor is expressed on monocytes and neutrophils (396,397) enabling these cells to respond to IL-6. Although IL-6 may not directly contribute to chemotaxis of monocytes and neutrophils it may enhance macrophage differentiation, phagocytic activity and increase antigen presentation (395,398). Levels of IL-6 are also reported to be increased in asthma, COPD and severe pneumonia patients (137,238,399,400). Increased levels of IL-6 measured in BAL fluid from asthmatics, are localised to cells including non-ciliated epithelial cells (137). Stimulated bronchial epithelial cells obtained from COPD patients are also reported to express increased levels of IL-6, which is not observed in cells from healthy patients (303).

The proinflammatory cytokines TNF-α and IL-1β are reported to increase IL-8 expression by airway epithelial cells (127,151,401). In a *C. trachomatis* infection model using cervical epithelial cells, both IL-8 and IL-6 were increased (402). However, it is suggested that the production of IL-1, which was also induced after infection, may be in part, responsible for the increased IL-8 and IL-6 production (402). We did not detect TNF-α or IL-1β protein released into BEAS-2B cell supernatant after stimulation of cells with either isolate of *C. pneumoniae* which is similar to results obtained after infection of A549 cells with *C. pneumoniae* (183). Therefore, the increase in IL-8 and IL-6 expression by BEAS-2B cells after exposure to *C. pneumoniae*, is unlikely to be induced by endogenous TNF-α or IL-1β.

It has previously been suggested that upregulation of cytokine expression by *C. pneumoniae* is mediated via modulation of signal transduction pathways. Both IL-8 and IL-6 contain binding sites for various transcription factors within their promoter regions, including NFκB (395,403,404). Furthermore, Jahn and colleagues showed that infection of BEAS-2B cells caused an increase in translocation of NFκB and subsequent increase in IL-8 gene expression (127). Endothelial cells infected with *C. pneumoniae* also show enhanced NFκB activity (141). Increased NFκB activity in endothelial cells are reported to be induced by two different chlamydial antigens, HSP-60 and polymorphic membrane proteins (24,254). Moreover, both antigens are also shown to increase IL-6 and IL-8 in endothelial cells (24,254). It is possible therefore, that the increase in IL-8 and IL-6 secretion in the currently documented studies, may be a result of increased NFκB activity mediated by chlamydial antigens such as HSP-60 or polymorphic membrane proteins. Antibodies to chlamydial HSP-60 have been detected in serum obtained from asthmatic subjects and inversely correlated to lung function (115,121). Studies have shown that there is some association between asthma and COPD and chronic or persistent *C. pneumoniae* infection (6,82,95,115,122). Recently, IFN-γ induced persistent infection of HEp-2 cells was shown to cause increased expression of HSP-60 (70). Therefore, persistent *C. pneumoniae* infection associated with chronic airways inflammatory disease may result in heightened expression of chlamydial proteins in airway epithelial cells leading to an increase in host transcription factor activity and modulation of cytokine expression.

The airway epithelium is also ideally situated to interact with other inflammatory cells, a process which is not only mediated by release of chemotactic factors but is also governed by the expression of cell surface markers. To date, there have been few publications examining the role of *C. pneumoniae* in the modulation of airway epithelial cell surface marker expression. Therefore, we also investigated the surface marker expression profile of BEAS-2B cells in response to *C. pneumoniae*. We examined the expression of a range of

surface markers involved in adhesion, antigen presentation and costimulation and found that the BEAS-2B cell line constitutively expressed the adhesion molecules ICAM-1 and CD58 as well as MHC Class I molecules. However, stimulation with either of the two *C. pneumoniae* isolates (IOL-207 and WA97001), did not significantly alter the level of expression of any of the molecules examined.

The results concerning ICAM-1 expression are in contrast to a previously published study using the same cell line, but using an alternative *C. pneumoniae* isolate, (GiD) that demonstrated a significant increase in ICAM-1 expression (127). The results in the presently documented studies may be specific to the two isolates used herein. Variation in the stimulating capacity of *C. pneumoniae* isolates has been shown in alternative systems using endothelial cells examining soluble ICAM-1 (391). Intercellular adhesion molecule-1 expression is also noted to be modulated by inflammatory cytokines such as TNF-α, IL-1β and IFN-γ (188,310) but, as mentioned previously, we did not detect significant levels of these proteins in cell supernatant which could partly explain why *C. pneumoniae* failed to cause an increase in ICAM-1 expression. The natural ligands for ICAM-1 are the lymphocyte function associated molecules including CD11a, CD11b and CD18, which are members of the β2 integrin family and are expressed on a number of leucocyte subsets (192). The expression of ICAM-1 on airway epithelial cells, is known to facilitate the interaction and adhesion of leucocytes to the epithelial cell layer (185). *C. pneumoniae* not only had no significant effect on the relative amount of ICAM-1 expression per cell, it also did not change the percentage of cells expressing these molecules. The percentage of BEAS-2B cells expressing ICAM-1 was maintained after stimulation with *C. pneumoniae*, thus allowing the epithelial cells to maintain their role in orchestrating interaction with other inflammatory cells mediated through ligation of ICAM-1.

The expression of a second adhesion molecule, CD58, on BEAS-2B cells in response to *C. pneumoniae* has not been previously investigated. There are few papers documenting the expression of CD58 on bronchial epithelium. We consistently detected CD58 expression on the BEAS-2B cell line which is consistent with a previous publication also finding constitutive CD58 expression by this cell line (197). Weak expression has also been detected in the respiratory tract including the bronchus and terminal bronchioles (194). As with ICAM-1 expression, *C. pneumoniae* stimulation of BEAS-2B cells failed to alter the percentage of cells expressing CD58 or the amount per cell they were expressing. The presence or absence of cytokines within BEAS-2B cell supernatant is unlikely to play a role in modulation of CD58 expression, as stimulation of cells with TNF-α or IFN-γ are not known to modulate CD58 expression (197,405). CD58 binds to CD2 which is expressed on T lymphocytes and NK cells (192,196). Therefore, the expression of CD58 on airway epithelial cells may be of importance in mediating adhesion of T lymphocytes and epithelial cells. Evidence which supports the notion that CD58 may mediate adhesion between non-haematopoietic cells and leucocytes is provided through studies utilising fibroblasts (406,407). In cocultures of fibroblasts and peripheral blood leucocytes, inhibitory antibodies to CD58 expressed on fibroblasts, significantly decreased the adhesion of T lymphocytes expressing CD2 (406). In addition, the expression of CD58 on airway epithelial cells and fibroblasts is also shown to be increased after infection with viruses but there is no evidence that this same phenomenon occurs with bacterial infection(407,408). Of interest, cytomegalovirus infection of fibroblasts which increases CD58 expression, also increases IL-6 and IL-8 expression (409). However, addition of recombinant IL-6 or IL-8 did not alter CD58 expression. This possibly suggests that infection with cytomegalovirus alters CD58 expression by a direct mechanism, which is not utilised by *C. pneumoniae*.

The adhesive mechanisms between airway epithelial cells and inflammatory cells, mediated by epithelial cell surface molecule expression, do not appear to be modulated by

C. pneumoniae. However, this does not prevent this opportunistic bacteria to alter the interaction between these cell types. Adhesion of eosinophils and airway epithelial cells are shown to be primarily mediated via β2 integrin expression on eosinophils rather than ICAM-1 expression by airway epithelial cells (185). Therefore, if *C. pneumoniae* is capable of regulating adhesion between epithelial cells and leucocytes, rather than modulating epithelial cell surface expression, its stimulating effects may be directed toward surface molecule expression on leucocytes.

We detected constitutive expression of MHC Class I expression by BEAS-2B cells, which is consistent with well accepted knowledge of MHC Class I expression by respiratory epithelial cells (188,190). However, upon stimulation of BEAS-2B cells with *C. pneumoniae*, the percentage of cells expressing MHC Class I molecules, nor the amount they express appeared to remain unchanged. The response of airway epithelial cells to *C. pneumoniae* in terms of antigen presentation has thus far, not been previously documented. Although airway epithelial cells are not the primary antigen presenting cells in lung defence mechanisms, it is possible that they play a primary role in initiation and regulation of tissue inflammation. It is interesting to note that the antigen presenting capacity of airway epithelial cells is not compromised by *C. pneumoniae*, which would allow the initiation of detection of the bacteria by the immune system. In a *C. trachomatis* model of infection utilising lung epithelial cells, MHC Class I expression is inhibited after infection, thought to be due to degradation of host cell transcription factors (8). The study by Zhong and colleagues, utilised HL cells (airway epithelial cells) but also showed similar inhibition in HeLa cells (cervical epithelium), and fibroblasts after infection with *C. trachomatis*. Together with the current studies, this may suggest that *C. pneumoniae* interacts with respiratory epithelial cells in a different manner to that seen with *C. trachomatis*.

In summary, the two *C. pneumoniae* isolates used in the currently documented studies have not been previously described in the context of airway epithelial cell immune responses. We have shown that *C. pneumoniae* stimulates airway epithelial cells to increase not only their secretion of IL-8 but also induces IL-6 expression. This study also suggests that the increased levels of IL-6 and IL-8 detected in chronic disease states, which are associated with *C. pneumoniae* infection, may in part be due to infection of the epithelium. Specific chlamydial antigens may be responsible for increased host cell transcription factor activity and subsequent modulation of cytokine networks within the epithelium. Therefore, the airway epithelium may initiate the inflammatory process to *C. pneumoniae* by releasing chemoattractants and mediate migration of cells such as neutrophils and monocytes to the site of infection (**Figure 3.9**). Airway epithelial expression of adhesion molecules ICAM-1 and CD58 are constitutively expressed and maintained after *C. pneumoniae* stimulation. This still permits the interaction between airway epithelial cells and inflammatory cells. Similarly, although the expression of MHC class I molecules are not altered by *C. pneumoniae*, the role that airway epithelial cells play in antigen presentation may be preserved. These processes may be important in allowing new progeny released from lysis of infected airway epithelial cells, to infect infiltrating inflammatory cells which may serve as a vehicle for systemic dissemination of *C. pneumoniae* and promote the development of long term chronic infection.

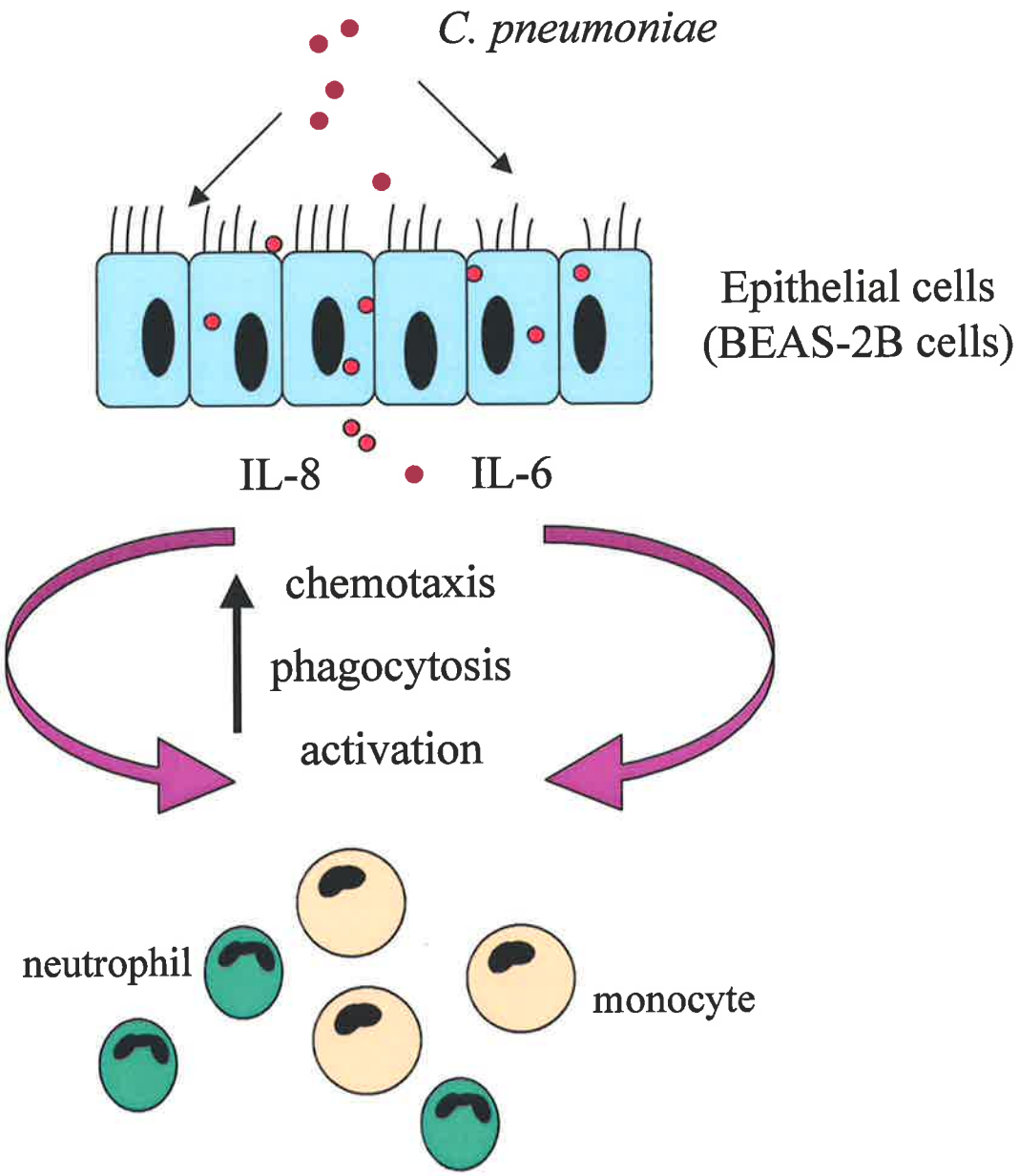


Figure 3.9: Airway epithelial cells increase IL-8 and IL-6 expression in response to *C. pneumoniae*.

CHAPTER 4

RESULTS

Cytokine response profiles of peripheral blood leucocytes stimulated with *C. pneumoniae*

Introduction

Monocytes are a rich source of both pro- and anti-inflammatory cytokines including TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-10 and IL-12 (220,223). These cytokines play important roles in regulating inflammation and immune responses to infection.

The inflammation associated with airways disease is not exclusively localised to the airways and respiratory system. Alteration to the levels of cytokines detected in peripheral blood, often reflect the inflammatory processes within the lungs. Further, peripheral blood cells from normal subjects, demonstrate heightened sensitivity to inflammatory stimuli (221,223). Stimulation of monocytes from COPD patients, with LPS, results in a significant increase in IL-1β, IL-6 and MCP-1 compared to cells obtained from healthy subjects (222,239). In asthmatic patients, the baseline level of GM-CSF expression by peripheral blood mononuclear cells is significantly higher than those in control patients and LPS stimulated cells from asthmatics produced more GM-CSF and IL-1β than cells from controls (222). In contrast, the level of IL-12 expression by whole blood monocytes is decreased in asthmatic patients after stimulation with LPS or *Staphylococcus aureus* (241-243). The levels of IL-10 have also been measured in asthmatic patients but there are conflicting results with some researchers reporting increases in patient groups and others observing no differences between patients and healthy controls (241,242). These studies show that cytokine levels detected in peripheral blood of patients with chronic respiratory disease show significant modulation of expression in comparison to healthy subjects.

It is well documented that freshly isolated human monocytes are susceptible to *C. pneumoniae* infection (129,216). Monocytes are professional phagocytes and antigen presenting cells and therefore play important roles in host defence and immune response processes. *C. pneumoniae* is primarily a respiratory pathogen but is also associated with other diseases such as atherosclerosis. It is hypothesised that after infection with *C. pneumoniae* within the respiratory tract, the bacteria is transmitted to the vasculature via granulocytes and alveolar macrophages, both of which are susceptible to infection with *C. pneumoniae* (130,218,349,357). Macrophages can cross the mucosal barrier and enter the circulation via lymphatic tissue as shown in animal models (218,410). Chlamydial DNA has been detected in circulating leucocytes of patients with COPD and other diseases including atherosclerosis (113,411). Researchers have also detected *C. pneumoniae* DNA in mononuclear cells of healthy blood donors (13,129,216,284). Once in the circulation, infected leucocytes, particularly monocytes are able to transmit the bacteria to other potential host cells including endothelium and smooth muscle cells (129,412,413). Monocytes are also able to be activated by bacterial infection and stimulated by bacterial products and may play a role in the initiation and promotion of inflammatory processes distal to the primary infection site (216,220,221,250,414).

A small number of publications have endeavoured to investigate the relationship between *C. pneumoniae* and cells of monocytic lineage. Monocytes are naturally adherent cells and this characteristic is often exploited in their purification and isolation. Peripheral blood mononuclear cells obtained by density centrifugation yields a mixed population of both monocytes and lymphocytes. By culturing peripheral blood mononuclear cells and by utilising the adherent properties of monocytes, these two cell types can be separated. However, the majority of studies investigating cytokine production by peripheral blood leucocytes in response to *C. pneumoniae*, have used peripheral blood mononuclear cells, without further purification. Kaukoranta-Tolvanen and colleagues demonstrated increased

secretion of TNF-α, IL-1β and IL-6 by peripheral blood mononuclear cells but the exact cell identity was not determined (251). The preparation of cells involved a standard procedure using an adhesion method of isolation but stated that only 42% of the cells were $CD14^+$ (a specific monocyte marker), leaving the question unanswered as to whether the cytokine production was predominantly secreted by monocytes or an alternative cell source, such as lymphocytes. The levels of TNF-α, IL-1β and IL-6 were greatly increased within 24 hours and showed a concentration dependent response. The cells used responded to both crude and purified preparations of *C. pneumoniae* and increased cytokine production. A later study by Netea and colleagues suggested that the source of *C. pneumoniae* induced TNF-α and IL-8 was peripheral blood monocytes, as purified lymphocytes released significantly less of each cytokine into cell supernatant, but no direct evidence was provided to confirm this (250). This study also illustrated that prior sonication of the *C. pneumoniae* preparation (semi-purified), assumed by the authors to kill the bacteria, was efficient at eliciting the observed cytokine response and was equivalent to that seen with live bacteria. In a later study by the same group, peripheral blood mononuclear cells stimulated with sonicated *C. pneumoniae* increased IL-10 secretion into cell supernatant but the exact cell source was left undetermined (249).

Although the above mentioned studies suggest that monocytes are the cell responsible for increased levels of cytokines detected in peripheral blood, none of the papers definitively illustrate this. Utilisation of monocytic cell lines has shown that purified preparations of *C. pneumoniae* increase the production of TNF-α, IL-1β and IL-6 secretion (215). A later study using a different monocyte cell line also illustrated increased levels of IL-6 and IL-10 cell culture supernatant after infection with *C. pneumoniae* (252). Although this paper also showed increased levels of IL-10 in supernatant from *C. pneumoniae* infected peripheral blood monocytes (determined to be 90 – 95% $CD14^+$ after adhesion), the preparation of *C. pneumoniae* in this paper was a crude preparation and the appropriate controls were not

used. In addition, these cells were cultured for up to 4 days to allow differentiation into macrophages before investigating the cytokine response to *C. pneumoniae*. Therefore, the specificity of the cytokine response of peripheral blood monocytes to *C. pneumoniae* infection in this paper remains uncertain.

Intranasal inoculation of mice with *C. pneumoniae* is reported to result in the detection of IL-10 and IL-12 in murine lungs and prompted researchers to investigate whether the expression of these cytokines was also increased by human peripheral blood mononuclear cells after infection with *C. pneumoniae* (248,415). Human peripheral blood mononuclear cells were infected with semi-purified preparations of *C. pneumoniae* and responded by increasing release of TNF-α, IL-10 and IL-12 (p40) into cell supernatant (248).

IL-12 and IL-10 are key regulators of the immune system. IL-12 is expressed as a heterodimer of two protein subunits, p35 and p40, forming a final complex of 70kDa (p70). It promotes the expression of IFN-γ, a key Th1 cytokine, by T lymphocytes and NK cells (416,417). IL-12 also promotes the expansion of IFN-γ producing cells and inhibits the development of IL-4 producing cells (294). The induction of IFN-γ results in increased Th1 type responses and cell mediated immunity and thereby promotes clearance of infection. It also inhibits Th2 associated cell processes and inhibits IL-10 expression by monocytes (227).

IL-10 is classified as a Th2 cytokine and has profound inhibitory effects on monocyte function. It inhibits the production of a variety of cytokines including TNF-α, IL-1β, IL-6, and IL-8 from monocytes (223). IL-10 also inhibits IL-12 expression by monocytes and leads to suppression of Th1 responses and a decrease in IFN-γ production by lymphocytes (226).

Therefore, the balance between IL-12 and IL-10 expression is critical to the pattern of cytokine expression profiles of monocytes and lymphocytes. In order to clear infection, cell

mediated immunity and promotion of Th1 responses is essential. Inhibition or decreased Th1 immune responses may lead to the development of chronic inflammation and persistent infections. It has been suggested that monocytes may act as a vehicle for systemic dissemination of *C. pneumoniae* (216,218,357). Failure to clear initial infection in the lungs before transfer of *C. pneumoniae* to monocytes, may not only enable transmission of the bacteria to sites distal to the initial infection but also contribute to inflammation at both the primary and secondary infection sites.

Therefore, infection with *C. pneumoniae* may contribute to altered cytokine levels in peripheral blood of individuals with chronic lung disease. However, a number of questions still remain about the profile of cytokines expressed by leucocytes and the exact cell source of these cytokines.

Aims

Therefore, the specific aims of the studies described in this chapter were:

a) To determine whether *C. pneumoniae* stimulates cytokine production by peripheral blood monocytes, in particular in the most physiologically relevant context, as a component of whole blood.

b) To investigate whether cytokines characterised as Th1 and Th2 cytokines (particularly IL-12 and IL-10) are also modulated by *C. pneumoniae*.

c) To compare the cytokine response of whole blood monocytes to different *C. pneumoniae* strains.

d) To investigate the involvement of chlamydial endotoxin (LPS) in induction of cytokines from whole blood mononuclear cells.

e) To determine whether *C. pneumoniae* stimulates lymphocyte cytokine production.

Methods

C. pneumoniae was propagated in HEp-2 cells, and harvested according to the protocol detailed in the methods chapter (Chapter 2). Uninfected cell lysate was also prepared in the same manner from uninfected HEp-2 cells.

To investigate the effect of *C. pneumoniae* on cytokine production by whole blood monocytes we utilised a stimulation protocol based on that described by Netea and colleagues (250). Venous blood was collected into tubes containing 20 units/ml sodium heparin as an anticoagulant, from volunteers, after obtaining informed consent, who were non atopic, non smokers with no history of respiratory disease.

A single serum sample from each volunteer was obtained to test for the presence of *C. pneumoniae* specific IgG and IgM antibodies using the MIF test (see Chapter 2) to establish whether volunteers had previously been exposed to the bacteria.

The remaining whole blood was diluted 1:1 with serum free cell culture medium and incubated overnight with *C. pneumoniae*, a comparable amount of uninfected cell lysate (which served as a negative control) or *E. coli* LPS (100ng/ml; which served as a positive control for monocyte stimulation), a combination of PMA and ION (25ng/ml and 1μg/ml respectively; which served as a positive control for lymphocyte stimulation) or cells were incubated in media alone which served as an unstimulated control. All samples were incubated with Brefeldin A (10μg/ml) which acted as a golgi block, inhibiting the release of cytokines from the cells, thus allowing accumulation of cytokines and detection by flow cytometry.

In studies examining the role of *C. pneumoniae* antigens in modulation of monocyte cytokine production, *C. pneumoniae* and uninfected cell lysate were sonicated for 10 minutes

(Bransonic 2200, Branson Ultrasonics, Danbury, CT) prior to overnight incubation with whole blood samples. In order to assess the contribution of chlamydial LPS to induction of cytokine expression by monocytes, stimuli were preincubated with polymixin B (final concentration 20mg/ml) for 2 hours at 37°C, or were heat inactivated at 100°C for 30 minutes. Polymixin B is an antibiotic that inhibits the activity of LPS derived from Gram negative bacteria (418). LPS is a thermostable molecule (419) therefore unaffected by heating and which enables us to determine whether heat sensitive proteins are involved in stimulating monocytes to express cytokines.

Intracellular cytokine production was measured using flow cytometry as described in Chapter 2 and shown in **Figure 2.10**. Whole blood monocytes were gated based on $CD14^+$ staining and side scatter characteristics (see **Figure 2.11**). These cells were then formatted in dot plots displaying intracellular staining for cytokines including TNF-α, IL-1α, IL-6, IL-8, IL-12 or IL-10. lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics (see **Figure 2.12**). These cells were then formatted in dot plots displaying intracellular staining for cytokines including TNF-α, IL-2, IFN-γ or IL-4.

Cellular viability was assessed by measuring total cell apoptosis by flow cytometric techniques previously described in Chapter 2. After incubating whole blood with the desired stimulus, total cellular apoptosis of monocytes and lymphocytes were determined by analysis of 7-AAD staining or a combination of 7-AAD and Annexin V FITC staining respectively.

Results

To determine whether volunteers had previously been exposed to *C. pneumoniae*, serum from each blood sample was analysed for *C. pneumoniae* specific antibodies using the MIF test. All serum samples were positive for *C. pneumoniae* specific IgG antibodies analysed at a single dilution of 1:16. The criteria used in the current study to identify individuals with pre-existing antibody or evidence of past infection is in accordance with previously defined criteria (3,84,88). These studies have defined past infection as a serum sample with an IgG titre ≥1:16 and <1:512. The results obtained from the MIF test suggest that all subjects participating in this study had evidence of past infection with *C. pneumoniae*.

Does C. pneumoniae stimulate whole blood monocytes to produce cytokines?

In order to confirm that monocyte cytokine production can be modulated by *C. pneumoniae*, we first needed to assess the capability of whole blood monocytes to express cytokines. Using flow cytometry, we validated our study design by investigating intracellular expression of TNF-α, IL-1α, IL-6 and IL-8 from whole blood monocytes after stimulation with *E. coli* LPS. In each case, less than 10% of unstimulated monocytes (selected based on $CD14^+$ staining and SSC characteristics) were positive for cytokine expression (**Figure 4.1**). As expected, exposure of the cells to *E. coli* LPS greatly increased expression and more than 50% of the cells were positive for cytokine expression. These results confirmed that whole blood monocytes ($CD14^+$) increase expression of TNF-α, IL-1α, IL-6 and IL-8 after stimulation with *E. coli* LPS and this treatment served as a positive control in subsequent experiments.

After finding that whole blood monocytes were responsive to *E. coli* LPS, our objective was to determine whether these same cytokines could also be induced by *C. pneumoniae*. We based our experimental design on the previously published observations of Netea and colleagues who stimulated peripheral blood mononuclear cells with a semi-purified

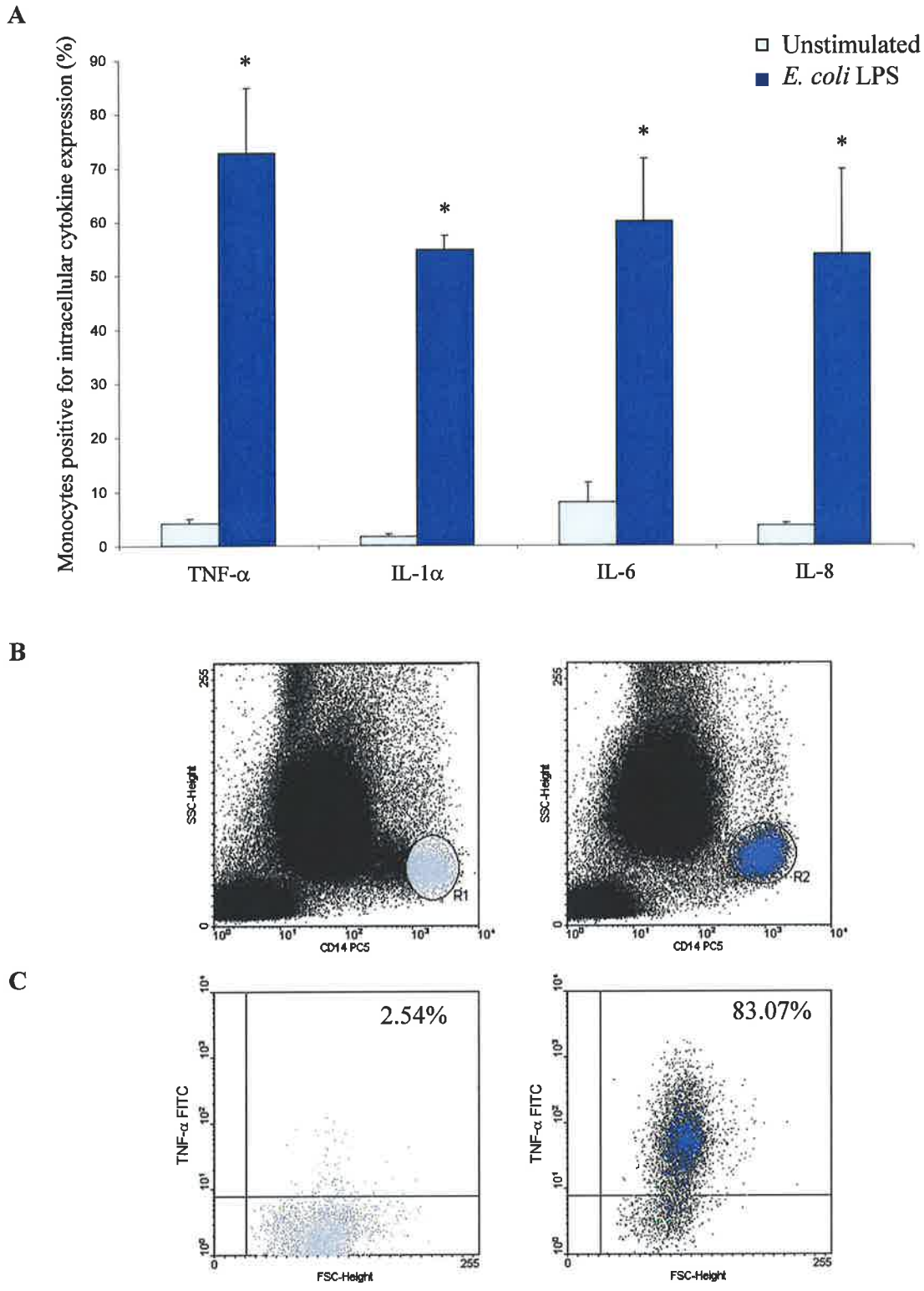


Figure 4.1: *E coli* LPS stimulates cytokine expression by whole blood monocytes.
A) Whole blood was incubated overnight with or without *E. coli* LPS (100ng/ml) in the presence of Brefeldin A (10μg/ml). Monocytes positive for cytokine expression are expressed as a percentage of monocytes, selected based on $CD14^+$ cells. Bars represent the mean ± SEM of 3 separate experiments, performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. The reciprocal of raw data for IL-8 expression was determined before applying the mixed ANOVA model for analysis. **B)** Dot plots showing examples of selection of monocytes based on $CD14^+$ staining and SSC characteristics. Note that R1 and R2 are different as it is well accepted to select the brightest staining cells. In addition, stimulated cells display different light scatter characteristics to resting cells. **C)** Dot plots showing an example of increased expression of TNF-α after overnight stimulation with *E. coli* LPS. Cells positive for cytokine expression were detected using fluorescent labelled monoclonal antibodies at the single cell level by flow cytometry. Percentages represent monocytes positive for TNF-α expression.

preparation of *C. pneumoniae* (250). However, we have specifically selected monocytes by flow cytometry techniques, enabling us to identify the cell source of cytokines induced by *C. pneumoniae*. The control treatment for *C. pneumoniae* stimulation of whole blood was included in the experimental design. This was a preparation of uninfected HEp-2 cells, (used for the propagation of *C. pneumoniae*) which underwent the same processing as infected cells. This yielded a preparation of uninfected cell lysate, which possibly contained remnants of the original HEp-2 cell line. Using flow cytometry we were able to detect significant increases in the percentage of monocytes expressing TNF-α, IL-1α, IL-6 and IL-8 after overnight stimulation with *C. pneumoniae* compared to uninfected cell lysate (**Figure 4.2**).

After demonstrating that *C. pneumoniae* stimulates cytokine expression by whole blood monocytes, we investigated whether *C. pneumoniae* alters the cytokine profile of host cells in terms of Th1 and Th2 cytokines. To explore this, whole blood was incubated overnight with *C. pneumoniae* and IL-12 and IL-10 cytokine production measured by flow cytometry. IL-12 expression was minimal in unstimulated monocytes but significantly increased by *E. coli* LPS (**Figure 4.3**). Uninfected cell lysate also increased the percentage of monocytes expressing IL-12, compared to unstimulated cells ($p<0.05$). Compared to the effect of uninfected cell lysate, overnight stimulation of whole blood with *C. pneumoniae*, resulted in a significantly elevated percentage of monocytes expressing IL-12 (**Figure 4.3**).

We also measured IL-10 expression by whole blood monocytes after overnight incubation with *C. pneumoniae*. We performed this experiment on three different individuals in triplicate and detected a small but significant increase in the percentage of monocytes expressing IL-10 compared to cells incubated with uninfected cell lysate (**Figure 4.4**).

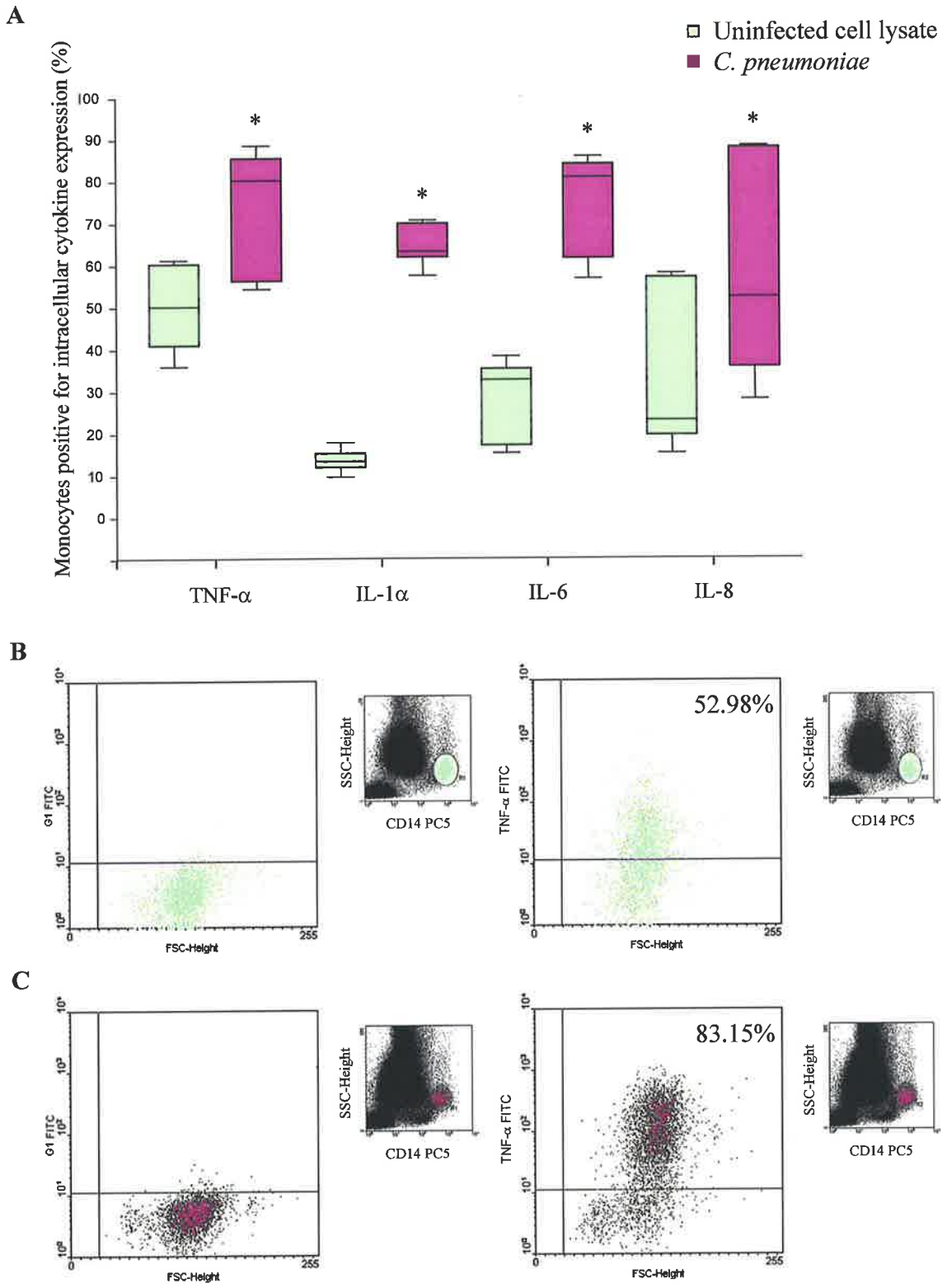


Figure 4.2: *C. pneumoniae* stimulates cytokine expression by whole blood monocytes.
A) Whole blood from 3 subjects was incubated overnight with *C. pneumoniae* ($1x10^6$ ifu/ml) or uninfected cell lysate in the presence of Brefeldin A (10μg/ml). Boxes represent the interquartile range of 3 separate experiments performed in triplicate. Whiskers represent the highest and lowest values, with the line across the box representing the median. * $p<0.05$, compared to uninfected cell lysate, mixed ANOVA. The raw data for cytokines TNF-α, IL-6 and IL-8 was log transformed before applying the mixed ANOVA model for analysis. Monocytes positive for cytokine expression are expressed as a percentage of monocytes selected based on $CD14^+$ cells (see insets B and C). Dot plots representative of isotype control antibody (left) or TNF-α FITC antibody (right) staining for cells treated with (**B**) uninfected cell lysate or (**C**) *C. pneumoniae*, $1x10^6$ ifu/ml. Cells positive for cytokine expression were detected using fluorescent labelled monoclonal antibodies by flow cytometry. Percentages represent monocytes positive for TNF-α expression. Note increased expression of TNF-α after incubation with *C. pneumoniae* compared to uninfected cell lysate.

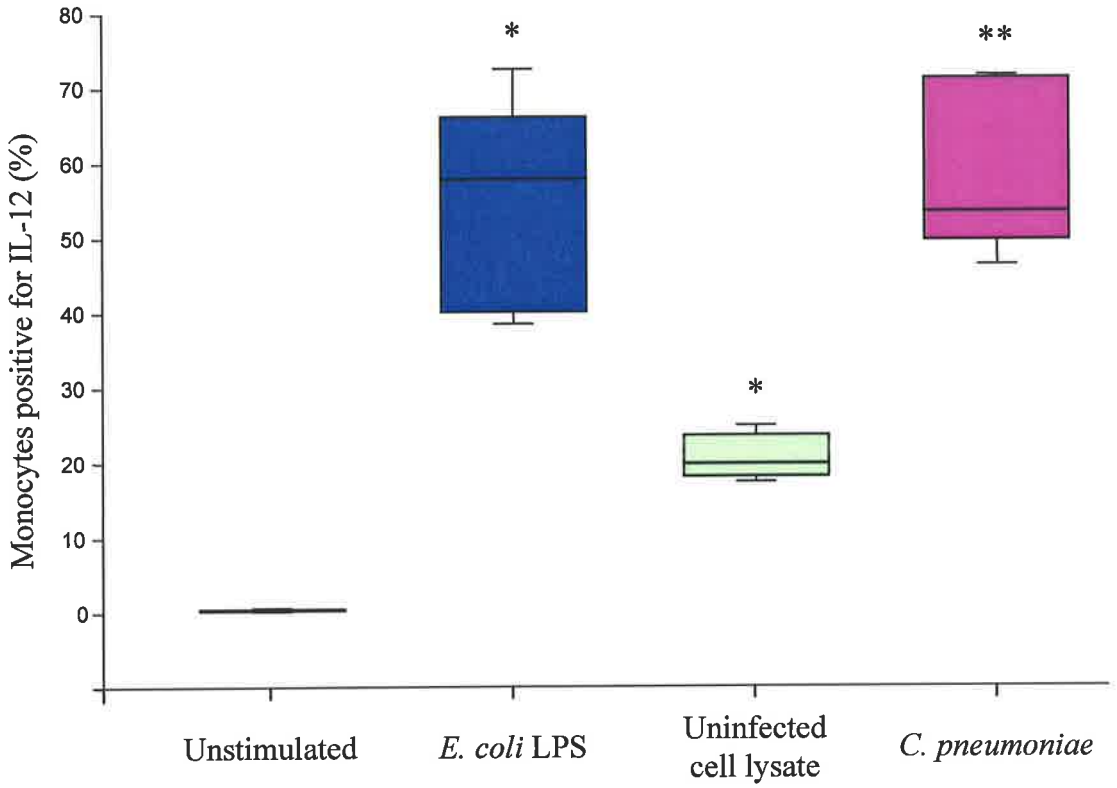


Figure 4.3: *C. pneumoniae* stimulates IL-12 expression by whole blood monocytes.
Whole blood was incubated overnight with *E coli* LPS (100ng/ml), uninfected cell lysate (sonicated) or *C. pneumoniae* ($1x10^6$ ifu/ml; sonicated) in the presence of Brefeldin A (10μg/ml). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and IL-12 positive cells were detected using an anti-human IL-12 PE labelled monoclonal antibody. Boxes represent the interquartile range of 3 separate experiments performed in triplicate. Whiskers represent the highest and lowest values, with the line across the box representing the median. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA. Raw data was transformed by square root before applying the mixed ANOVA model for analysis.

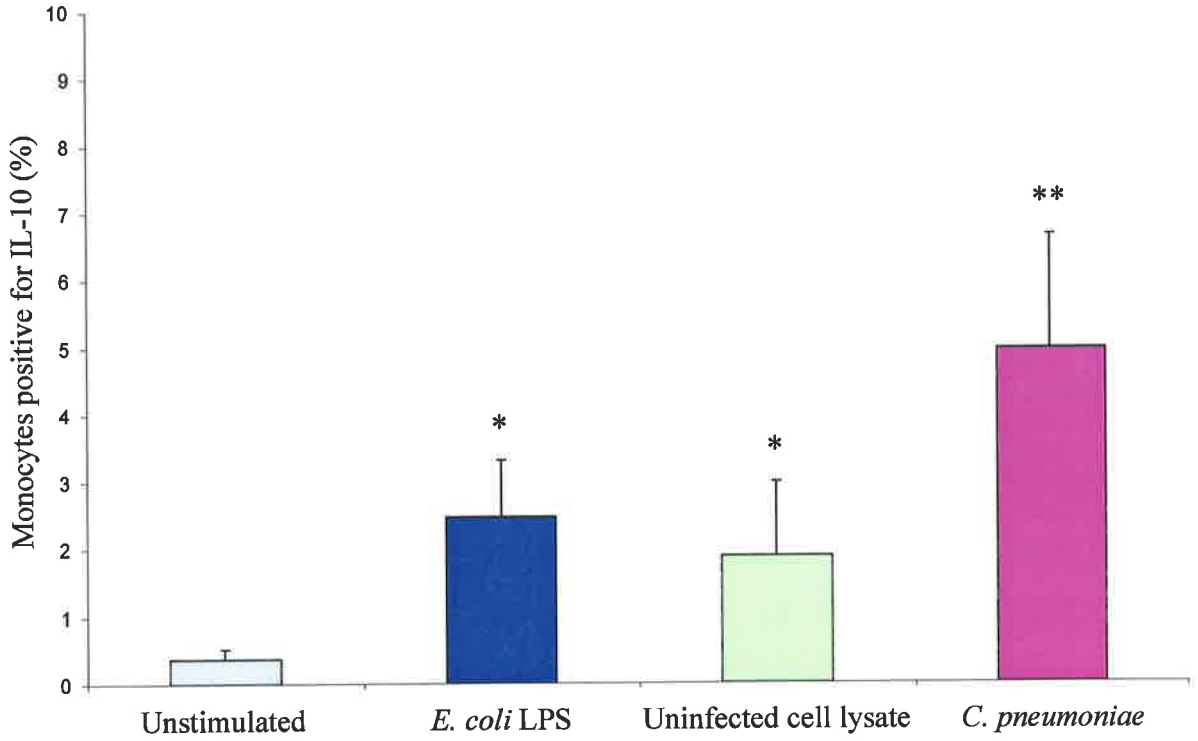


Figure 4.4: *C. pneumoniae* (IOL-207) increases IL-10 expression by whole blood monocytes.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (sonicated) or *C. pneumoniae* ($1x10^6$ ifu/ml; sonicated) in the presence of Brefeldin A (10μg/ml). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and IL-10 positive cells were detected using an anti-human IL-10 PE labelled monoclonal antibody. Bars represent the mean ± SEM of 3 separate experiments performed in triplicate. * $p<0.05$ compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

Do different C. pneumoniae isolates induce similar patterns of monocyte cytokine production?

To determine whether monocyte cytokine induction by *C. pneumoniae* is strain specific we evaluated a second *C. pneumoniae* isolate, WA97001 (386). Unlike the IOL-207 isolate (employed in all studies until this point) which was originally isolated from a patient with trachoma, WA97001 was isolated from a nasopharyngeal specimen (386). Due to the different origin of each of the two isolates we performed a comparative study investigating whether the two isolates induced different cytokine profiles in whole blood monocytes.

Whole blood was incubated with the different *C. pneumoniae* isolates and intracellular cytokine production measured by flow cytometry. Due to the occasional stimulating capacity of uninfected cell lysate, presumably due to the presence of HEp-2 cell debris, different concentrations of each isolate were prepared using uninfected cell lysate as a diluent to maintain similar background protein concentrations.

Overnight stimulation with IOL-207 or WA97001 resulted in a significant increase in the percentage of monocytes expressing TNF-α, IL-1α, IL-6 and IL-8, when compared to cells incubated with uninfected cell lysate (**Figure 4.5A-D**). When cells were incubated with equivalent concentrations of IOL-207 and WA97001 ($1x10^5$ ifu/ml), we observed that the WA97001 isolate stimulated the monocytes to a greater extent and resulted in a significantly higher percentage of monocytes expressing cytokines compared to those stimulated with IOL-207. Both isolates exhibited a dose response relationship, with cytokine expression decreasing with exposure to decreased concentration of *C. pneumoniae*.

Of more interest was the response of whole blood monocytes to the two *C. pneumoniae* isolates when measuring intracellular IL-12 and IL-10 production. As can be seen in **Figure 4.5E**, both isolates increased the percentage of monocytes expressing IL-12 compared to uninfected cell lysate. When equivalent concentrations of each isolate were used, a similar

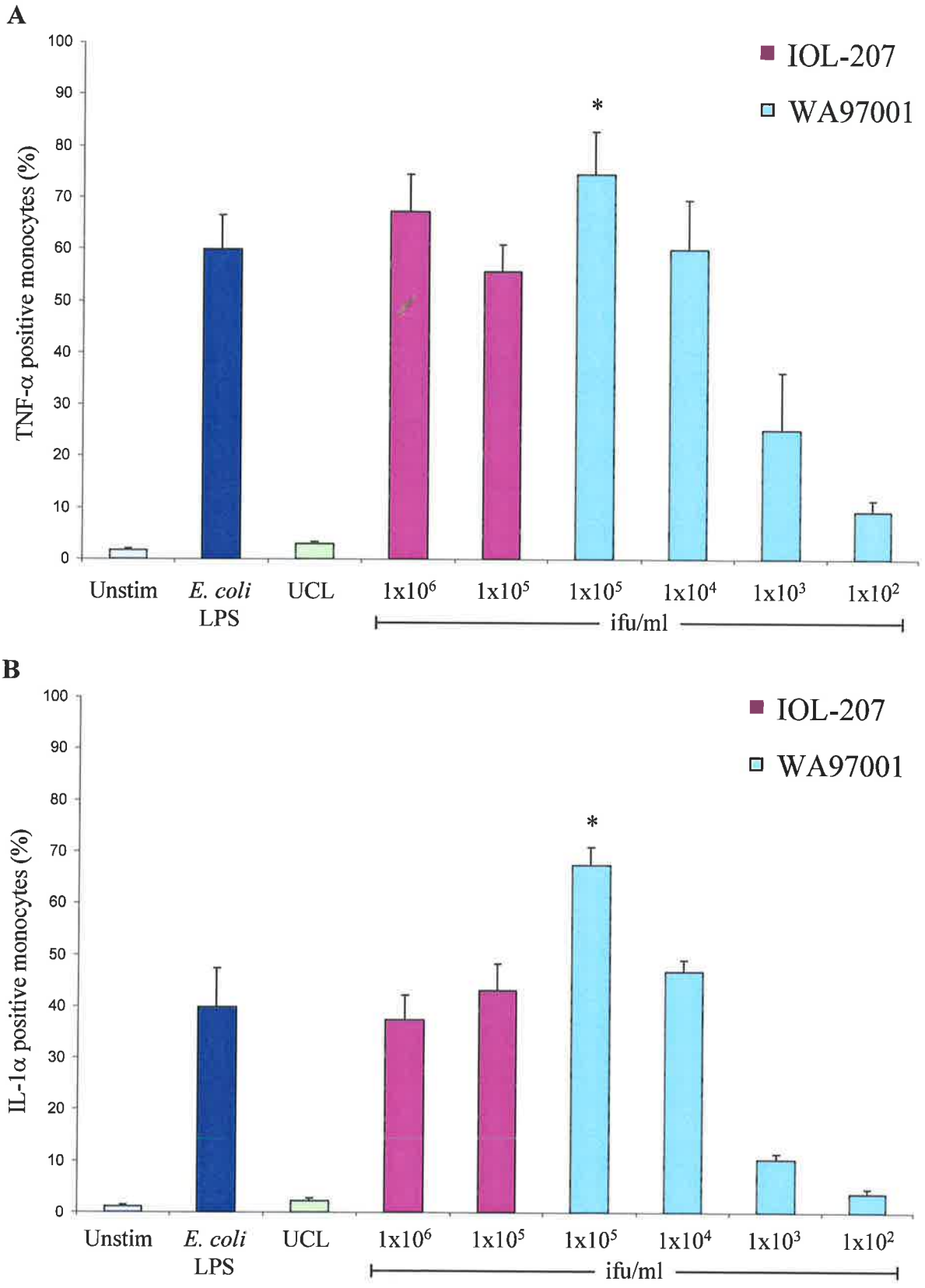


Figure 4.5: Comparison between *C. pneumoniae* isolates IOL-207 and WA97001 in stimulating TNF-α and IL-1α expression by whole blood monocytes.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207 or WA97001 isolates), at varying concentrations. All overnight incubations were performed in the presence of Brefeldin A (10 μg/ml) to allow measurement of intracellular cytokine production. Monocytes were gated based on $CD14^+$ antibody staining and TNF-α (**A**)and IL-1α (**B**) positive monocytes were measured using anti-human FITC labelled monoclonal antibodies by flow cytometry. Bars represent the mean ± SEM of 3 separate experiments performed in duplicate. * $p<0.05$ compared to IOL-207 ($1x10^5$ ifu/ml), mixed ANOVA.

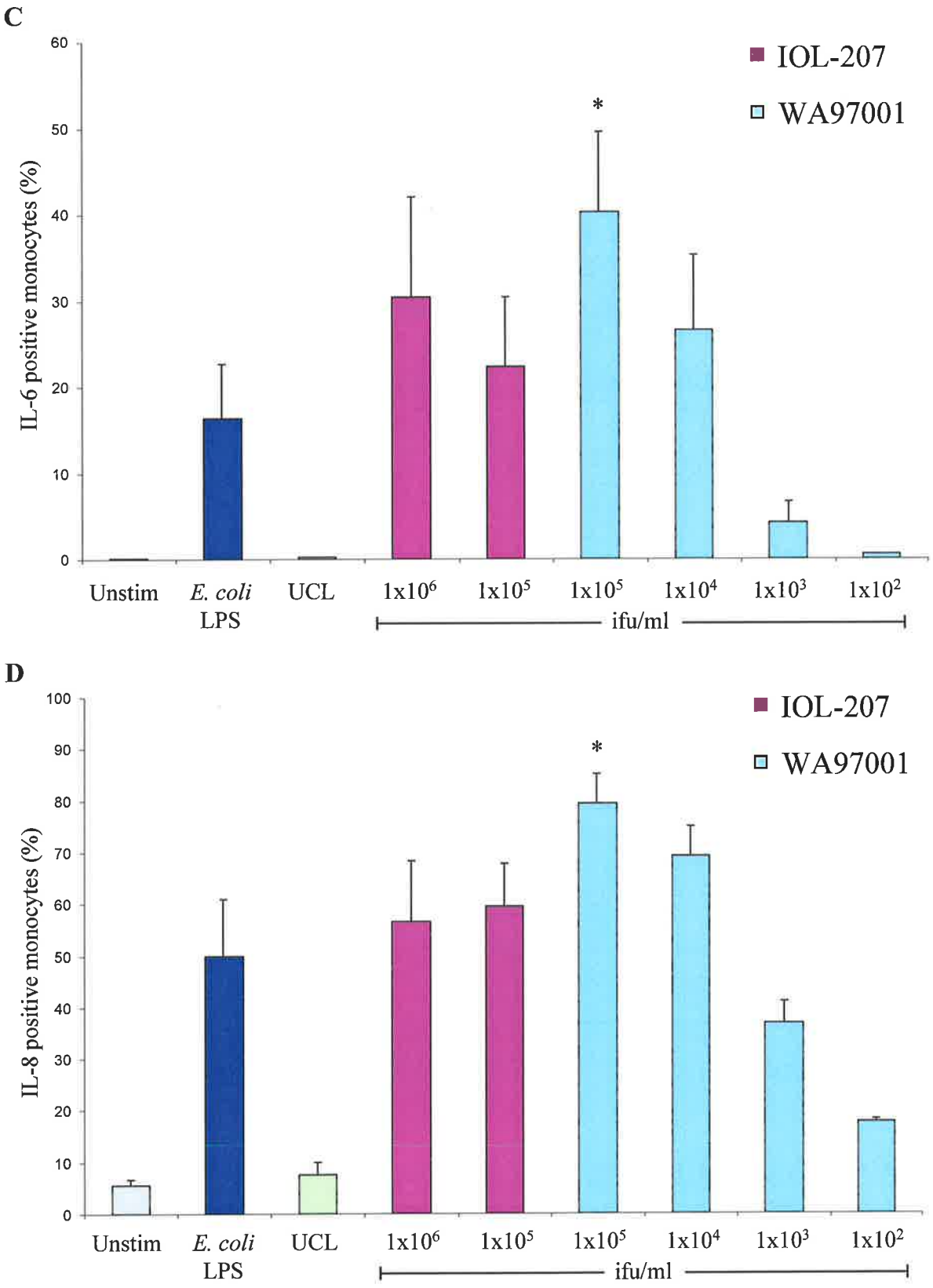


Figure 4.5: Comparison between *C. pneumoniae* isolates IOL-207 and WA97001 in stimulating IL-6 and IL-8 expression by whole blood monocytes.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207 or WA97001 isolates), at varying concentrations. All overnight incubations were performed in the presence of Brefeldin A (10 μg/ml) to allow measurement of intracellular cytokine production. Monocytes were gated based on $CD14^+$ antibody staining and IL-6 (**C**) and IL-8 (**D**) positive monocytes were measured using anti-human PE or FITC labelled monoclonal antibodies by flow cytometry. Bars represent the mean ± SEM of 3 separate experiments performed in duplicate. * $p<0.05$ compared to IOL-207 ($1x10^5$ ifu/ml), mixed ANOVA. Raw IL-8 data was cubed before applying the mixed ANOVA model for analysis.

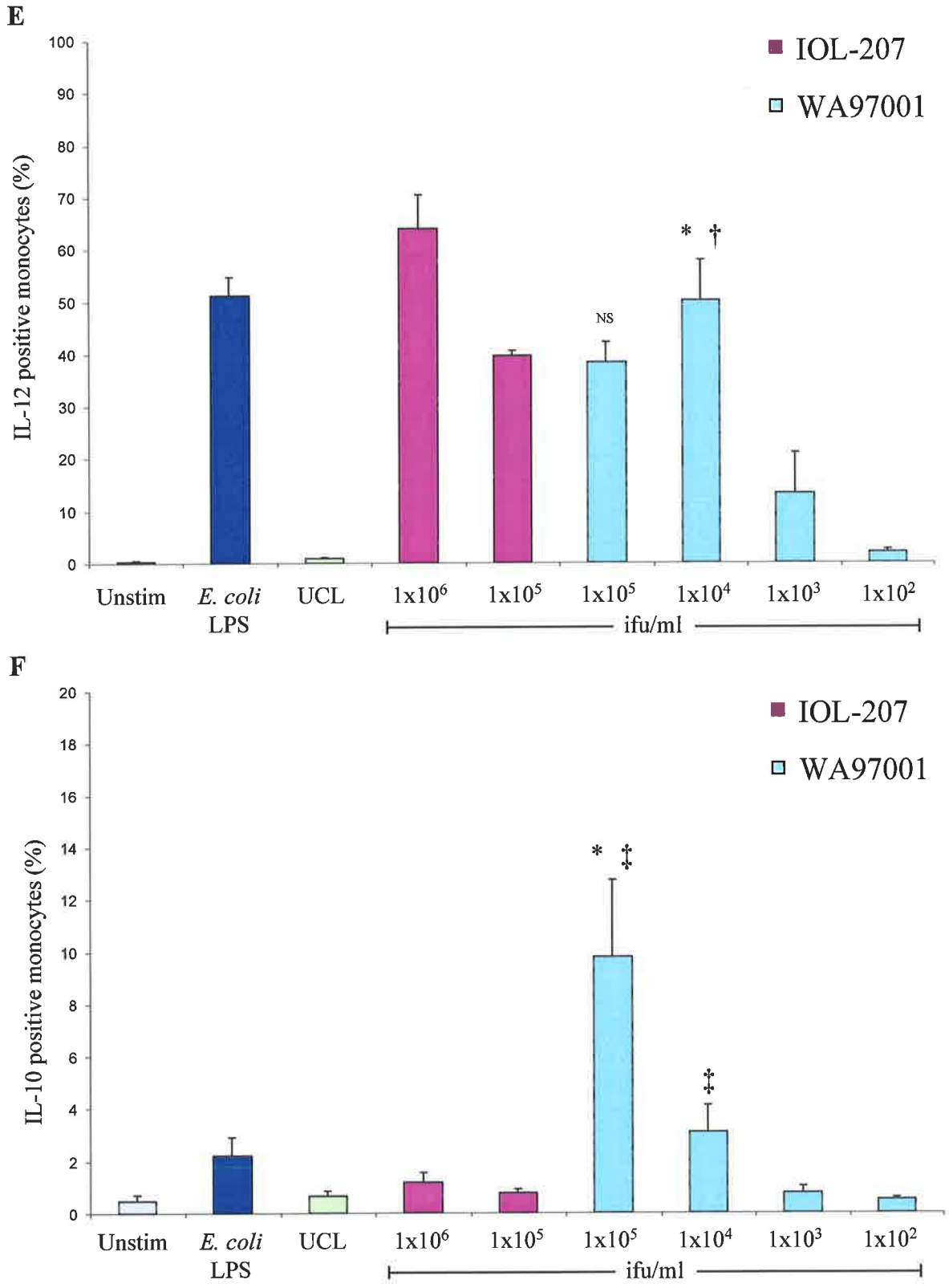


Figure 4.5: Comparison between *C. pneumoniae* isolates IOL-207 and WA97001 in stimulating IL-12 and IL-10 expression by whole blood monocytes.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207 or WA97001 isolates), at varying concentrations. All overnight incubations were performed in the presence of Brefeldin A (10 µg/ml) to allow measurement of intracellular cytokine production. Monocytes were gated based on $CD14^+$ antibody staining and IL-12 (**E**) and IL-10 (**F**) positive monocytes were measured using anti-human PE labelled monoclonal antibodies by flow cytometry. Bars represent the mean ± SEM of 3 separate experiments performed in duplicate. ^{NS} not significant compared to IOL-207 ($1x10^5$ ifu/ml); * $p<0.05$ compared to IOL-207 ($1x10^5$ ifu/ml); † $p<0.05$ compared to WA97001 ($1x10^5$ ifu/ml); ‡ $p<0.05$ compared to UCL, mixed ANOVA. Raw data for IL-10 was log transformed before applying the mixed ANOVA model for analysis.

level of induction of IL-12 expression was observed. Although increasing concentrations of WA97001 initially increased IL-12 expression by whole blood monocytes, the expression was subsequently decreased at the highest concentration used ($1x10^5$ ifu/ml; $p<0.05$).

In conjunction with this data, the higher concentrations of WA97001 ($1x10^4$ ifu/ml and $1x10^5$ ifu/ml) significantly increased the percentage of whole blood monocytes expressing IL-10 compared to uninfected cell lysate incubation (**Figure 4.5F**). This is in contrast to stimulation with IOL-207 which did not signficantly induce IL-10 expression by whole blood monocytes at the concentrations tested.

As the WA97001 isolate of *C. pneumoniae* caused a decrease in the percentage of monocytes expressing IL-12 at the highest concentration used, we explored this phenomenon further with the IOL-207 isolate. Whole blood was stimulated overnight with different concentrations of *C. pneumoniae* (IOL-207) or comparable concentrations of uninfected cell lysate (negative control). Monocytes positive for IL-12 expression were detected using flow cytometric techniques. As can be seen in **Figure 4.6**, the pattern of IL-12 expression after stimulation with the IOL-207 isolate was similar to that which was observed as for WA97001. When cells were stimulated with the IOL-207 *C. pneumoniae* strain at $1x10^7$ ifu/ml, the percentage of monocytes expressing IL-12 was significantly increased compared to uninfected cell lysate (UCL). However, upon stimulation with the highest concentration of *C. pneumoniae*, the percentage of monocytes expressing IL-12 was significantly decreased compared to cells stimulated with $1x10^6$ ifu/ml.

To assess whether the decreases seen in IL-12 expression were due to a cytotoxic effect of *C. pneumoniae*, total monocyte apoptosis was measured by flow cytometry. The average percentage of monocytes exhibiting positive staining with 7-AAD, after stimulation with uninfected cell lysate was not significantly different compared to unstimulated cells

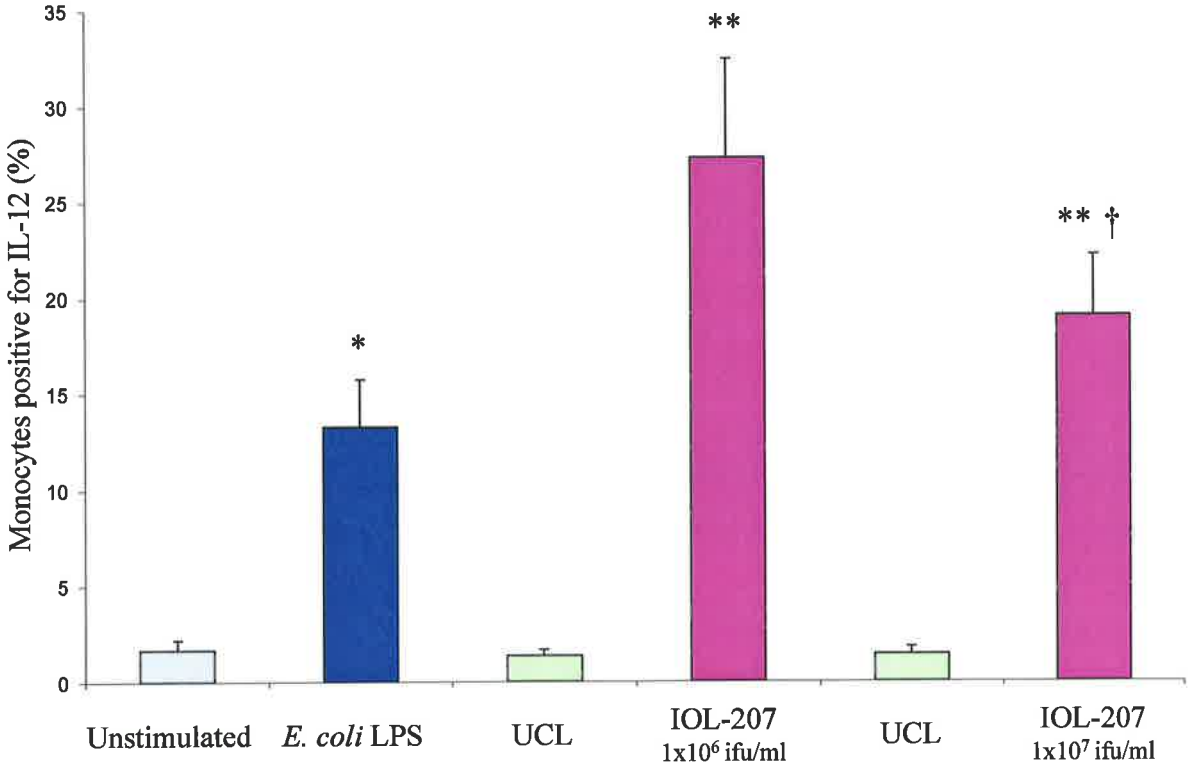


Figure 4.6: *C. pneumoniae* (IOL-207) decreases IL-12 expression at high doses by whole blood monocytes. Whole blood was stimulated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL; comparable concentration to *C. pneumoniae*) or different concentrations of *C. pneumoniae* (IOL-207). All overnight incubations were performed in the presence of Brefeldin A (10μg/ml) to allow measurement of intracellular cytokine production. Monocytes were gated based on $CD14^+$ antibody staining and IL-12 positive monocytes were measured using an anti-human PE labelled IL-12 antibody. Bars represent the mean ± SEM of 5 separate experiments performed in duplicate. * $p<0.05$ compared to unstimulated cells; ** $p<0.05$, compared to uninfected cell lysate (UCL); † $p<0.05$ compared to IOL-207, $1x10^6$ ifu/ml, mixed ANOVA.

(**Table 4.1**). Stimulation with the lower concentration of *C. pneumoniae* ($1x10^6$ ifu/ml) did not significantly alter the percentage of monocytes staining for 7-AAD. However, the highest concentration of *C. pneumoniae* ($1x10^7$ ifu/ml) did cause a small increase in 7-AAD staining which was significantly increased to 7.2%, compared to cells stimulated with a comparable concentration of uninfected cell lysate (3.2%).

Percentage of monocytes positive for 7-AAD staining					
Subject	**Unstimulated**	**UCL10[a]**	***C. pneumoniae*[b] ($1x10^6$ ifu/ml)**	**UCL100**	***C. pneumoniae* ($1x10^7$ ifu/ml)**
1	1.20 ± 0.18	1.50 ± 0.38	2.04 ± 0.27	2.62 ± 0.65	8.56 ± 0.31
2	1.72 ± 0.26	0.71 ± 0.24	0.25 ± 0.11	0.88 ± 0.15	0.83 ± 0.46
3	7.56 ± 0.65	2.42 ± 0.44	3.94 ± 1.40	6.10 ± 0.56	12.21 ± 1.33
Mean ± SEM	3.49 ± 2.04	1.54 ± 0.50	2.08 ± 1.06[NS]	3.20 ± 1.54	7.20 ± 3.36*

Table 4.1: Effect of *C. pneumoniae* on monocyte viability.
Whole blood was stimulated overnight with the desired stimulus before analysis of monocytes staining for 7-AAD by flow cytometry. Data represents 3 separate experiments performed in triplicate. [a] UCL, Uninfected cell lysate; [b] *C. pneumoniae*, IOL-207; [NS] Not statistically different compared to appropriate concentration of UCL, mixed ANOVA. * $p<0.05$, compared to UCL100, mixed ANOVA. The square root of the raw data was used for statistical analysis.

Do C. pneumoniae antigens play a role in the modulation of monocyte cytokine production?

After demonstrating that *C. pneumoniae* induces the production of TNF-α, IL-1α, IL-6, IL-8, IL-12 and IL-10 in whole blood monocytes, we next asked whether chlamydial antigens play a role in this stimulation. In the experiments performed by Netea and colleagues, the preparation of *C. pneumoniae* was first sonicated to kill the bacteria and determine whether the host cell response was different to that seen with live bacteria (250). We also adopted this approach and used the same protocol to kill the bacteria, and incubated whole blood with sonicated preparations of *C. pneumoniae* or uninfected cell lysate, and compared them with blood that was incubated with *C. pneumoniae* that had not been sonicated.

We measured intracellular cytokine production by flow cytometry and found that sonication of the *C. pneumoniae* preparation did not significantly change the cytokine profile of whole blood monocytes compared to those that were stimulated with *C. pneumoniae* which had not

been sonicated (**Figure 4.7**). The results suggest that live bacteria are not required to induce a cytokine response from peripheral blood monocytes and that chlamydial antigens alone may be responsible for stimulation of monocytes. We addressed this question in the next series of experiments.

Does chlamydial LPS stimulate monocyte cytokine production?

We have shown that *C. pneumoniae* induces the production of a number of cytokines. We have also demonstrated that *E. coli* LPS is a strong stimulator of monocyte cytokines and have therefore used it as a positive control in each experiment. *C. pneumoniae* also has a bacterial endotoxin, which raised the possibility that the induction of cytokines by *C. pneumoniae* was in fact due to its LPS component. To determine whether *C. pneumoniae* LPS contributed to the induction of cytokines from whole blood monocytes, we performed a series of inhibition studies. Polymixin B is a natural antibiotic that binds to the lipid A portion of lipopolysaccharide in the cell wall of gram negative bacteria (420). Therefore, polymixin B was preincubated with *C. pneumoniae* preparation (also *E. coli* LPS and uninfected cell lysate) prior to overnight stimulation of whole blood. Lipopolysaccharide is also known to be heat stable (419), therefore some stimuli were also heat inactivated prior to overnight incubation.

Incubation of blood with polymixin B alone had no significant effect on the percentage of monocytes expressing IL-1α (**Figure 4.8**). As expected, IL-1α expression induced by *E. coli* LPS was significantly decreased by pretreatment of *E. coli* LPS with polymixn B. As LPS is thermostable, heating *E. coli* LPS prior to stimulation of monocytes, had no effect on the percentage of IL-1α positive cells. Pretreatment of uninfected cell lysate with polymixin B or heating, also had no effect on the number of monocytes positive for IL-1α. In contrast, *C. pneumoniae* induced IL-1α expression was significantly reduced after pretreatment with polymixin B. Heat inactivation of *C. pneumoniae* also significantly decreased the percentage

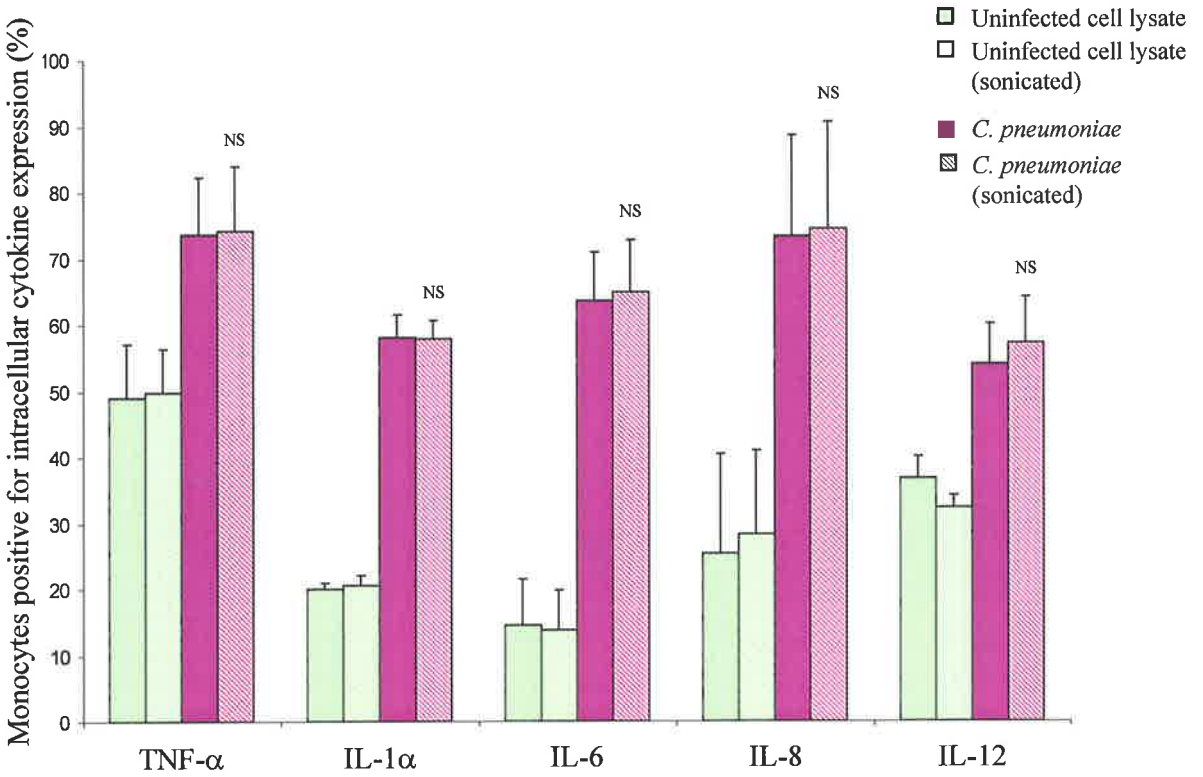


Figure 4.7: Effect of sonicated *C. pneumoniae* on intracellular cytokine production by whole blood monocytes. Whole blood monocytes were stimulated overnight with uninfected cell lysate or *C. pneumoniae*, with or without sonication prior to adding to cell cultures, in the presence of Brefeldin A (10μg/ml). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and intracellular cytokines detected using fluorescent labelled anti-human monoclonal antibodies and are expressed as the percentage of positive monocytes. Bars represent the mean ± SEM of 3 separate experiments performed in triplicate. ^{NS} Not statistically significant compared to *C. pneumoniae* that was not sonicated, mixed ANOVA.

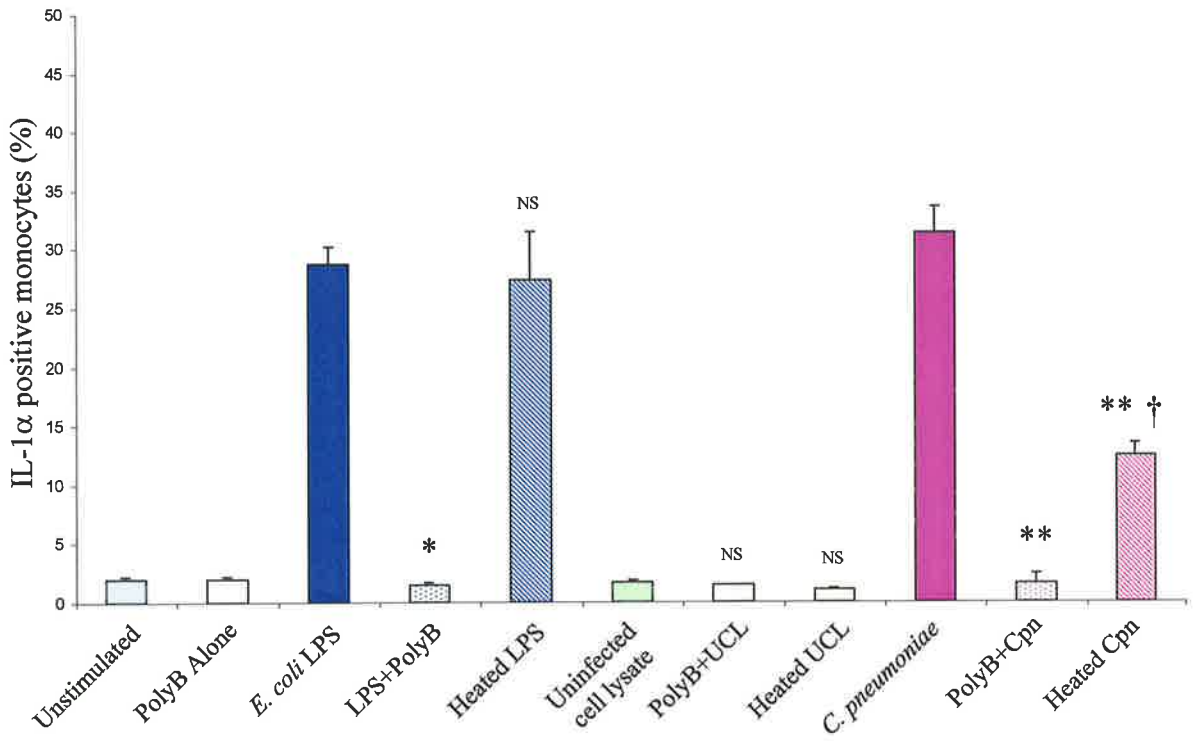


Figure 4.8: Chlamydial LPS and heat sensitive chlamydial antigens increase monocyte expression of IL-1α. Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate or *C. pneumoniae* (Cpn; $1x10^6$ ifu/ml) alone (block coloured bars) or preparations that had been preincubated with polymixin B (PolyB; final concentration 20mg/ml) for 2 hours at 37°C (dotted bars), or heat inactivated at 100°C for 30 minutes (diagonal bars). All overnight incubations were performed in the presence of Brefeldin A (10 μg/ml) to allow measurement of intracellular cytokine production. IL-1α positive monocytes were detected using an anti-human FITC labelled monoclonal antibody and measured using flow cytometry. Bars represent the mean ± SEM of the percentage of monocytes positive for IL-1α expression. Graph is representative of one of three separate experiments, performed in triplicate. * $p<0.05$, compared to *E. coli* LPS; ^{NS} $p<0.05$ compared to uninfected cell lysate; ** $p<0.05$, compared to *C. pneumoniae*; † $p<0.05$ compared to uninfected cell lysate ANOVA.

of monocytes expressing IL-1α. However, heat treatment of *C. pneumoniae* did not completely abolish the percentage of monocytes expressing IL-1α and was still significantly increased compared to uninfected cell lysate. Thus, these studies suggest that the stimulatory effect of *C. pneumoniae* may largely be due to *C. pneumoniae* LPS, but that a heat labile component may also make some contribution.

A similar study was performed to determine the contribution of *C. pneumoniae* antigens to IL-12 expression by whole blood monocytes. Incubation of blood with polymixin B alone had no significant effect on the percentage of monocytes expressing IL-12, compared to unstimulated cells to which no additional stimulus had been added (**Figure 4.9**). Similar to the data obtained for IL-1α, the increase in IL-12 expression by monocytes after *E. coli* LPS stimulation was completely abolished after pretreatment with polymixin B. Heating *E. coli* LPS prior to incubation with blood, had no significant effect on the percentage of monocytes expressing IL-12. Pretreatment of uninfected cell lysate with polymixn B or heating had no effect on the number of monocytes positive for IL-12. *C. pneumoniae* induced IL-12 expression was significantly inhibited after pretreatment with polymixin B. Heat treatment of *C. pneumoniae* also caused a small but significant decrease in the number of IL-12 positive monocytes which was similar to that observed for IL-1α, but to a lesser extent.

Similar inhibition studies were performed measuring intracellular production of TNF-α by whole blood monocytes. These experiments were completed on three individuals but the results were not consistent between subjects and therefore the chlamydial antigens responsible for the induction of TNF-α expression by whole blood monocytes could not be further elucidated.

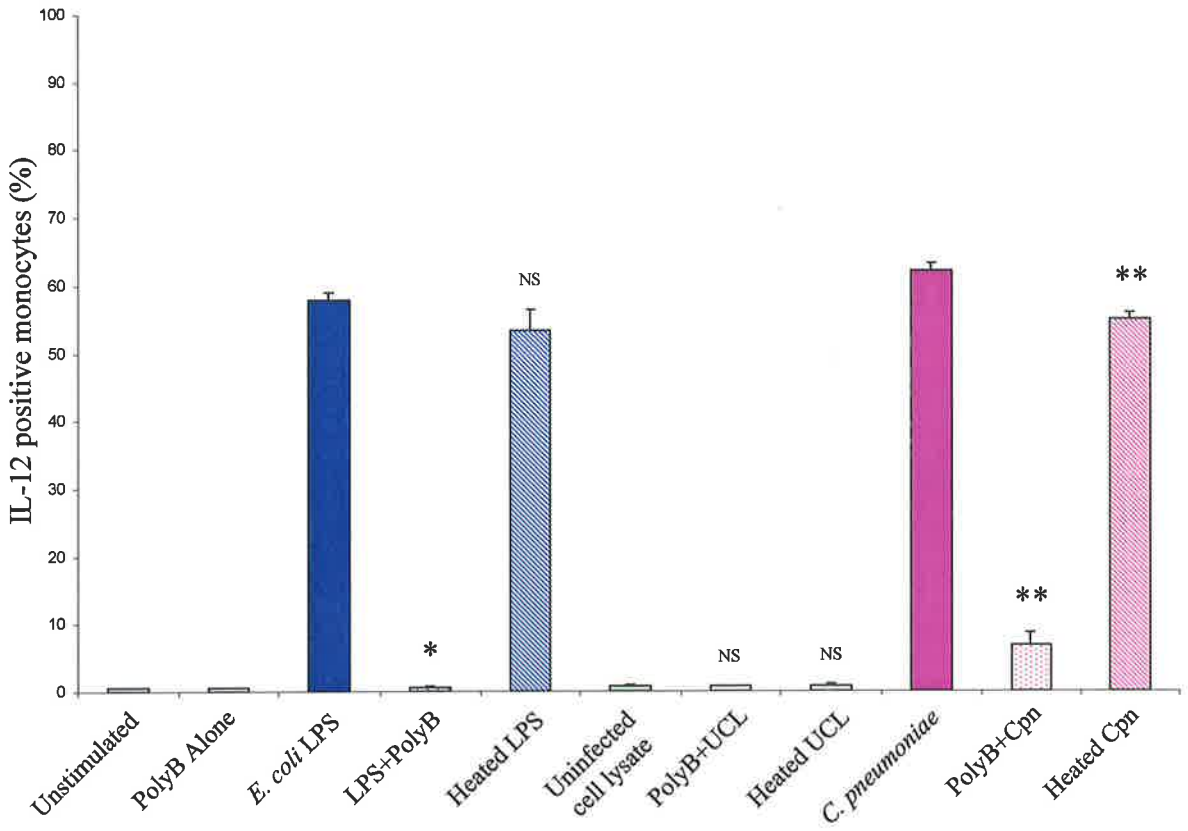


Figure 4.9: Chlamydial LPS increases monocyte expression of IL-12.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate or *C. pneumoniae* ($1x10^6$ ifu/ml) alone (block coloured bars) or preparations that had been preincubated with polymixin B (final concentration 20mg/ml) for 2 hours at 37°C (dotted bars), or heat inactivated at 100°C for 30 minutes (diagonal bars). All overnight incubations were performed in the presence of Brefeldin A (10 μg/ml) to allow measurement of intracellular cytokine production. IL-12 positive monocytes were detected using an anti-human PE labelled monoclonal antibody and measured using flow cytometry. Bars represent the mean ± SEM of the percentage of monocytes positive for IL-12 expression. Graph is representative of one of three separate experiments, performed in triplicate. * $p<0.05$, compared to *E. coli* LPS; ^{NS} $p<0.05$ compared to uninfected cell lysate; ** $p<0.05$, compared to *C. pneumoniae*, ANOVA.

Although both *C. pneumoniae* isolates employed in these studies induced monocyte IL-10 expression, the percentages of monocytes positive for IL-10 were quite low and the contribution on *C. pneumoniae* antigens to this induction could not be determined.

Does C. pneumoniae stimulate lymphocytes to produce cytokines?

Following the investigation of monocyte cytokine expression after stimulation with *C. pneumoniae* and consistently demonstrating its ability to induce the expression of a variety of cytokines, we questioned whether *C. pneumoniae* also stimulated lymphocyte cytokine production. The combination of phorbolmyristate acetate and ionomycin are commonly used as stimuli for T cell cytokine production. We first investigated whether *C. pneumoniae* directly stimulated lymphocytes to express cytokines. Whole blood was collected and processed similar to experiments investigating monocyte cytokine production using PMA and ION as a positive control, as opposed to *E. coli* LPS. Overnight stimulation with PMA and ION significantly increased the percentage of lymphocytes expressing TNF-α, IL-2 and IFN-γ (**Figure 4.10A**). However, *C. pneumoniae* did not directly stimulate lymphocytes to express any of the cytokines analysed.

We next asked whether *C. pneumoniae* primed lymphocytes to express cytokines upon subsequent stimulation with PMA and ION. Cells were initially incubated overnight with PMA and ION, *C. pneumoniae* or uninfected cell lysate and were then incubated for a further 24 hours with or without additional PMA and ION. As shown previously, lymphocyte cytokine production was not induced by *C. pneumoniae* stimulation alone (**Figure 4.10B**). lymphocyte expression of TNF-α, IL-2 and IFN-γ was increased in samples to which PMA and ION was added after 24 hours of prior stimulation (**Figure 4.10C**). However, the increase in cytokine expression was not significantly different compared to cells which were originally stimulated with uninfected cell lysate (or unstimulated cells). Therefore, the observed increase in cytokine production in cells initially stimulated with *C. pneumoniae* was most likely due to the subsequent incubation with PMA and ION. Cells that were initially

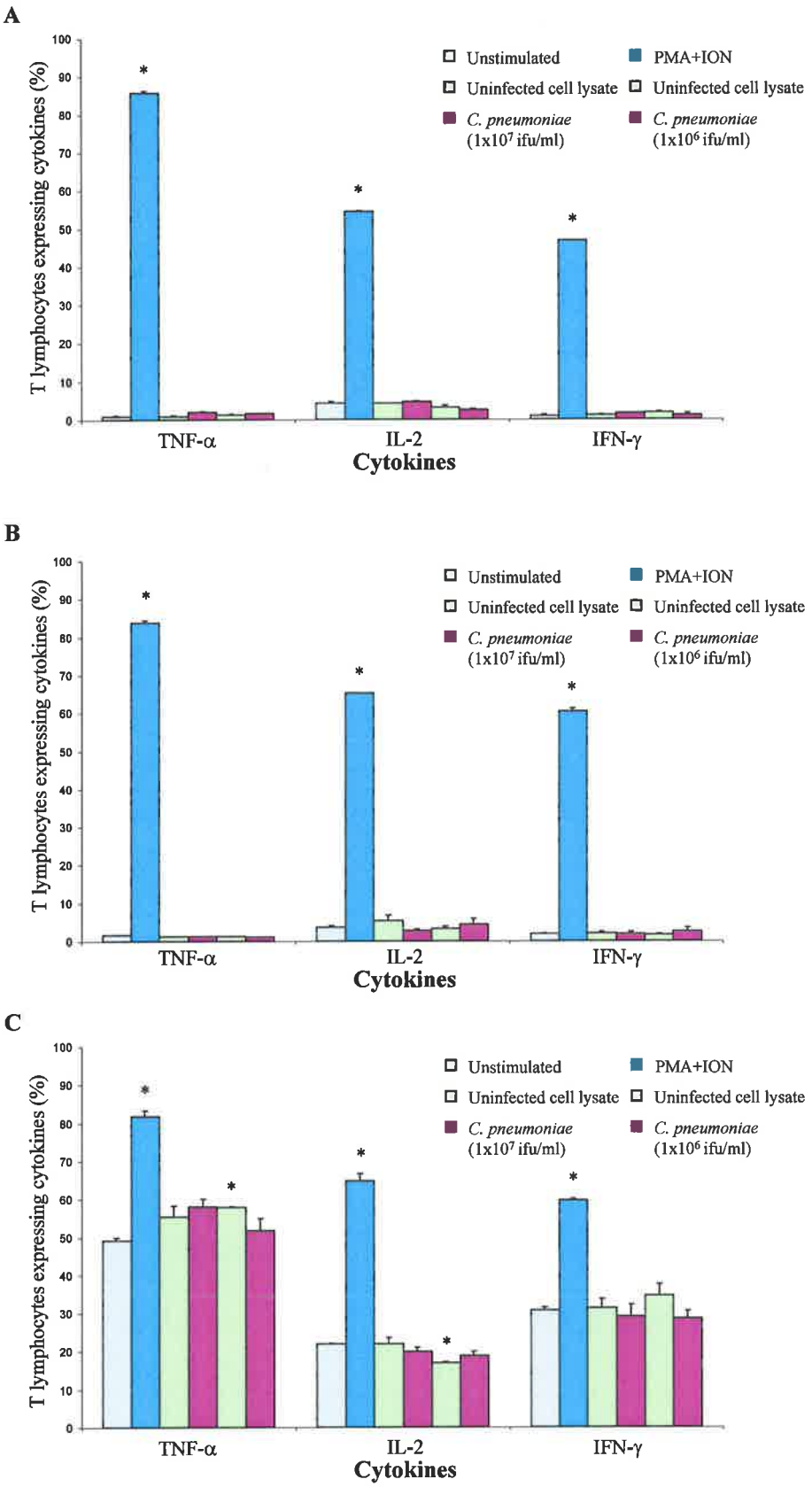


Figure 4.10: *C. pneumoniae* has no affect on cytokine production by whole blood T lymphocytes.
Whole blood was initially incubated overnight with PMA (25ng/ml) and ION (1μg/ml), uninfected cell lysate (concentration comparable to *C. pneumoniae* concentration), or *C. pneumoniae* (IOL-207), in the presence of Brefeldin A (10μg/ml), to allow measurement of intracellular cytokine production. **A)** Direct stimulation of blood with desired stimulus for 24hrs. **B)**. Direct stimulation of blood with desired stimulus for 48hrs. **C)** Stimulation of blood with desired stimulus for 24hrs prior to additional stimulus of PMA and Ionomycin for a further 24hrs. T lymphocytes were gated based on $CD3^+$ antibody staining and T lymphocytes positive for cytokine production were detected using anti-human FITC or PE labelled monoclonal antibodies by flow cytometry. Bars represent the mean ± SEM of 1 experiment, performed in duplicate. * $p<0.05$ compared to unstimulated, linear regression. Raw data was transformed before performing statistical analyses.

stimulated with PMA and ION and then incubated with additional PMA and ION, maintained their cytokine expression which was significantly higher than that of unstimulated cells.

We also investigated the expression of IL-4 by lymphocytes after stimulation of whole blood with *C. pneumoniae*. However, under all conditions tested we did not detect any IL-4 positive lymphocytes.

Discussion

Our findings here demonstrate that peripheral blood monocytes produce a range of cytokines in response to *C. pneumoniae* infection. We have confirmed that *C. pneumoniae* stimulation of peripheral blood mononuclear cells results in increased expression of a number of cytokines including TNF-α, IL-1α, IL-6, IL-8, IL-12 and IL-10 from peripheral blood mononuclear cells. This is consistent with previously published reports, however, the exact cell source of the inflammatory mediators in these previous studies was uncertain (248,250,251). These previous studies had used peripheral blood mononuclear cells isolated from whole blood by density gradient centrifugation, a cell population that contains both monocytes and lymphocytes (421). The most common protocol for separating monocytes and lymphocytes in this preparation is to exploit the adherent characteristics of monocytes and culture peripheral blood mononuclear cells before washing away non-adherent cells (lymphocytes). Kaukoranta-Tolvanen and colleagues did in fact purify this population by culturing the cells and removing lymphocytes, but stated that as little as 42% of the cells were monocytes, the remainder being lymphocytes (251). Therefore, the exact cell source of the cytokine secretion observed in response to *C. pneumoniae* stimulation, cannot definitively be determined. More evidence was provided in the study by Netea and colleagues, suggesting that TNF-α and IL-8 production was predominantly expressed by monocytes (250). They showed that isolated lymphocytes (>95% pure) produced significantly less of these cytokines compared to peripheral blood mononuclear cells, in response to *C. pneumoniae*. There was no additional attempt however, to isolate monocytes and confirm this suggestion. The most convincing evidence that human monocytes respond to *C. pneumoniae* stimulation in terms of cytokine production, comes from a study that purified peripheral blood mononuclear cells by adherence yielding a population that contained 90% monocytes and showed an increase in IL-10 and IL-12 (248). The focus of this paper was not however, to investigate the host cell response to *C. pneumoniae* in terms of cytokine production and no other cytokines were investigated. Therefore, although these studies have suggested that monocytes may respond

to *C. pneumoniae* by modulating their production of cytokines, there are some questions remaining about the identity of cells within the peripheral blood mononuclear cell population most commonly used in these studies and which cytokines are involved. The studies presented herein have now clarified the situation.

The use of flow cytometry in the current studies allowed us to select monocytes based on their expression of the classic surface marker for monocytes CD14 by using fluorescent labelled monoclonal antibodies together with their side scatter characteristics. The cells expressing high amounts of CD14 could then be gated and evaluated for cytokine expression through staining with a second fluorescent labelled antibody. This enabled us to conclude with confidence that the cellular source of intracellular cytokine expression was peripheral blood monocytes.

We also demonstrated that whole blood monocytes increase IL-12 expression after *C. pneumoniae* stimulation. An unexpected finding during this section of study, was that uninfected cell lysate also significantly increased the percentage of monocytes expressing IL-12 compared to unstimulated cells, which demonstrates the importance of using an appropriate control for the *C. pneumoniae* preparation. Previous studies measuring cytokines released into the supernatant of the U937 cell line or freshly isolated monocytes stimulated with a crude preparation of *C. pneumoniae* show increased levels of IL-6 and IL-10 by ELISA (252). However, the control treatment in these experiments were unstimulated cells, not treated with any additional stimulus, such as uninfected cell lysate. Therefore, the contribution to induced cytokine expression of cellular debris from the original host cells, cannot be excluded. Although the peripheral blood mononuclear cell preparation used in the studies performed by Kaukoranta-Tolvanen was a mixed population of monocytes and lymphocytes, their control stimulation consisted of a mock control, and from cells in which *C. pneumoniae* was originally cultured (251). Their study showed an increase in TNF-α,

IL-1β, IL-6 and IFN-α production after exposure to *C. pneumoniae* compared to mock stimulation. Of interest is that all four cytokines were clearly detectable after stimulation with their mock control supporting the results that we observed after stimulation with uninfected cell lysate. We are therefore confident that in the current studies, the induction of IL-12 and other cytokines by whole blood monocytes are due to *C. pneumoniae* stimulation and not non-specific host cell responses to cellular debris which may be present in the bacterial preparation.

We found that the percentage of monocytes expressing IL-12 was initially increased by both isolates used in the current studies. Stimulation with WA97001 caused an increase in IL-12 expression at a concentration of *C. pneumoniae* as low as $1x10^3$ ifu/ml. IL-12 expression peaked at $1x10^4$ ifu/ml and $1x10^6$ ifu/ml for the WA97001 and IOL-207 isolates respectively. In similar studies, a dose response relationship was observed with concentrations of up to $1x10^5$ ifu/ml using the TW-183 isolate (248). However, we also observed a subsequent decrease in IL-12 expression at higher bacterial concentrations. This phenomenon was observed for both isolates which showed a decrease in IL-12 expression at the highest concentrations used, $1x10^7$ ifu/ml for IOL-207 and $1x10^5$ ifu/ml for WA97001. In all cytokines examined in these studies where an equal concentration of both isolates were used to stimulate monocytes, the percentage of cells staining positive for the cytokines was significantly greater when the WA97001 isolate was used. We are confident that these differences are not due to technical variation encountered during quantitation of chlamydial stocks. Titration of both isolates were performed in parallel experiments in triplicate as described in Chapter 2. The number of inclusions counted within each triplicate set of coverslips was reproducible and showed minimal variability. We are therefore confident that monocyte cytokine expression profiles induced by the two different *C. pneumoniae* isolates are not due to variation in original determination of *C. pneumoniae* concentration in stock preparations of the bacteria. Interestingly, when comparable amounts of both *C. pneumoniae*

isolates were used, IL-12 expression was increased to the same extent. However, the WA97001 strain caused a decrease in IL-12 expression at a concentration 100-fold less than that observed with the IOL-207 isolate. Together this data suggests a tendency for the WA97001 *C. pneumoniae* isolate to exhibit an increased potency in host cell immune responses, compared to the IOL-207 isolate. The results in the previous chapter also support an increased potency of the WA97001 *C. pneumoniae* isolate when investigating the relationship with airway epithelial cells and *C. pneumoniae* infection. Other studies have also noted variability in the activity of different *C. pneumoniae* strains when analysing inflammatory mediator release and ability to stimulate chemotaxis of cells such as monocytes and neutrophils (356,391).

Due to the decrease in IL-12 at higher concentrations of *C. pneumoniae* we examined the viability of whole blood monocytes after stimulation. We were able to demonstrate that the decrease in cytokine production was not attributed to cell death (ie apoptosis). Although there was a statistically significant increase in the percentage of monocytes staining for 7-AAD, used as an indicator of apoptosis, after stimulation with a high concentration of *C. pneumoniae*, over 90% of the cells were still viable. In addition, there are numerous reports of *C. pneumoniae* inhibiting apoptosis in various cell types including monocytes (214,248,422). Therefore, the decrease in intracellular cytokine production in monocytes after stimulation with higher concentrations of *C. pneumoniae,* is unlikely to be attributed to apoptosis.

At the highest concentration of the nasopharyngeal isolate, WA97001, used we also observed that the percentage of monocytes expressing IL-10 was significantly increased. Our data is consistent with observations made by others who have measured IL-10 released into the supernatant of peripheral blood mononuclear cells after infection with *C. pneumoniae* (248,249,252). The majority of these studies used different strains of *C. pneumoniae* and

showed increased IL-10 secretion with lower concentrations of *C. pneumoniae* in their investigations. However, Geng and colleagues used the TW-183 isolate at $1x10^5$ ifu/ml, the same concentration as used in our study, and measured increased IL-10 secretion by radioimmunoassay in purified human monocytes (248). Flow cytometric measurement of intracellular cytokine expression is measured in terms of the percentage of cells staining positively for cytokines so it is difficult to compare the level or extent of the response, with other studies that have measured secreted proteins by ELISA or radioimmunoassays. Our results are consistent with previously published results measuring IL-10 production by cells of monocytic origin and the advantage of measuring intracellular cytokine expression in whole blood leucocytes has enabled us to simultaneously examine the cell source of the cytokines investigated.

The pattern of IL-12 and IL-10 expression by monocytes stimulated by *C. pneumoniae* may play an important role in acute versus chronic infection. Lower concentrations of *C. pneumoniae* initially caused a predominance of IL-12 expression, a Th1 cytokine, with no evidence of IL-10 production, (Th2 cytokine). This was followed by a subsequent decrease in IL-12 at higher *C. pneumoniae* concentrations, coupled together with increased IL-10 expression. IL-12 and IL-10 are two key regulatory cytokines in the immune response. The decrease in IL-12 expression could lead to diminished cell mediated immunity, regulated by IL-12. As IL-12 plays a major role in the regulation of cell mediated immunity, *C. pneumoniae* may compromise the host cell response to infection. IL-12 stimulates the production of IFN-γ by T lymphocytes and natural killer cells, also increasing their cytotoxic activity, thereby increasing the immune systems ability to fight infection (289). *C. pneumoniae* induced IL-10 expression by monocytes could also contribute to this cascade by inhibiting IL-12 production by peripheral blood mononuclear cells and consequently decreased IFN-γ production (226). In addition, the role of IL-10 induced by *C. pneumoniae* may also be important in the development of persistent infection. *C. pneumoniae* inhibits

apoptosis in monocytes and is suggested to be mediated via IL-10 (248). Inhibition of host cell apoptosis may facilitate the survival, propagation and dissemination of *C. pneumoniae*, which may lead to chronic infection.

The interaction between monocytes and lymphocytes is a key process in mounting an efficient immune response. The studies presented here, indirectly support the interaction of accessory cells, such as monocytes, with lymphocytes. We showed that *C. pneumoniae* does not directly stimulate lymphocyte cytokine production. The inability of T cells to increase IFN-γ in response to *C. pneumoniae* in our system, may be due to Brefeldin A preventing monocytic IL-12 release into cell supernatant. Similarly, although IL-10 decreases IFN-γ, IL-2 and IL-4 production by T cells, the inhibition of secretion of soluble factors from monocytes by Brefeldin A would prevent these interactions from occurring (159,287). The production of cytokines by T cells in response to T lymphocytes may require additional signals from accessory cells that are not provided by *C. pneumoniae* alone. The induction of IFN-γ by *C. pneumoniae* in other systems measuring cytokine levels in cell supernatant may be a secondary response due to IL-12 expression and not via direct stimulation by the bacteria (250,415).

Murine studies of *C. pneumoniae* infection support a role for IL-12 in Th1 mediated responses. Treatment of mice infected with *C. pneumoniae* with inhibitory antibodies to IL-12 results in increased bacterial titres suggesting an important function for this Th1 cytokine in the clearance of acute infection (415). In part, the protective mechanism of IL-12 against infection may be mediated through induction of IFN-γ which is also decreased after inhibition of IL-12 (415). Later studies by the same group demonstrated that neutralisation of IL-10 in peripheral blood mononuclear cells stimulated with *C. pneumoniae* results in an increase in IL-12 (248). However, our experimental design utilising flow cytometry to measure intracellular cytokine expression allows us to observe single cell populations without

any influence of other cytokines induced by *C. pneumoniae*. The presence of Brefeldin A in this system, inhibits the release of cytokines into the cell supernatant and allows accumulation of protein within the cell. Therefore the reduction of IL-12 observed in our system is unlikely to be attributed to the corresponding increase in IL-10. This suggests that *C. pneumoniae* may promote Th2 responses and cause a reduction in Th1 responses independently. Consequently the ability of *C. pneumoniae* to cause an imbalance between IL-12 and IL-10 may promote ineffective clearance of infection and allow the progression of infection into a chronic nature, associated with disease states.

The question of Th1 and Th2 responses to chlamydial infection has been addressed in *C. trachomatis* infection. In patients with severe conjunctival scarring as a result of *C. trachomatis* infection, peripheral blood mononuclear cells increase the production of the Th2 cytokine IL-4 in response to chlamydial antigens (305). This pattern was in comparison to control subjects who responded by increased production of the Th1 cytokine IFN-γ. In addition, the importance of Th1 cell mediated immunity in clearance of infection is shown in patients with trachoma. In ocular infection due to *C. trachomatis*, patients who recovered from infection demonstrated increased Th1 type responses in comparison to patients who showed evidence of persistent chlamydial infection (304). Similarly in female patients with a history of pelvic inflammatory disease and a history of repeated infection with *C. trachomatis*, IFN-γ release into plasma is reduced after stimulation of peripheral blood with the chlamydial antigen HSP-60, compared to patients who had did not have a history of multiple *C. trachomatis* infection (306).

Similar observations have been noted in reactive arthritis patients which is a disease triggered by infection with bacteria such as *C. trachomatis*. Mononuclear cells isolated from synovial fluid of reactive arthritis patients with *C. trachomatis* infection, produced high amounts of IL-10 and comparatively low amounts of IFN-γ and TNF-α (423). Furthermore, this study

demonstrated that the reduction in IFN-γ expression could be attributed to the inhibitory effects of IL-10 mediated via suppression of IL-12 expression. Also in support of a Th2 inflammatory response to chlamydial infection, cells within the synovial membrane of reactive arthritis patients, show an increased percentage of IL-4 positive cells compared to those expressing IFN-γ (423). These studies support the hypothesis that chlamydial infection promotes Th2 type cytokine profiles and that Th1 responses are important in resolving infections.

It is possible that the pattern of cytokine levels in chronic lung diseases such as asthma may be a reflection of the relationship between host cells and *C. pneumoniae*. This notion is supported by evidence of decreased levels of IL-12 and IFN-γ by peripheral blood mononuclear cells in asthmatics (241-243). The levels of IL-10 in asthmatics is however, controversial with some reporting increased expression levels whilst others report significantly less IL-10 in asthmatics compared to non-asthmatic patients (231,241). In some cases the imbalance of Th1 and Th2 specific cytokines seen in chronic diseases such as asthma may in part be a result of *C. pneumoniae* infection and provide an environment that decreases cell mediated immunity. A diminished immune response may lead to a failure to clear the initial infection and thereby promote the development of persistent infection.

The modulation of cytokine expression by whole blood monocytes stimulated by *C. pneumoniae* in the current studies is unlikely to be influenced by endogenous cytokine expression. There are however, a number of ways in which *C. pneumoniae* may regulate cytokine production. We were able to demonstrate that prior sonication of the *C. pneumoniae* preparation did not alter the cytokine response profile of *C. pneumoniae* stimulated whole blood monocytes. Consistent with our observations, Netea and colleagues observed comparative expression of TNF-α and IL-1β between *C. pneumoniae* preparations that underwent prior sonication and those that did not (250). They suggested that the regulation of

cytokines was mediated by acellular components of the bacteria. We investigated this phenomenon by performing a series of inhibition studies. The data suggests that induction of IL-1α and IL-12 expression by *C. pneumoniae* stimulated whole blood monocytes were possibly mediated by different mechanisms. We found that IL-1α expression was decreased when *C. pneumoniae* was preincubated with polymixin B which inhibits bacterial endotoxin (418). However, we also found that heat inactivation of *C. pneumoniae* also resulted in a significant decrease in IL-1α expression. Taken together, this data suggests that *C. pneumoniae* induces IL-1α expression mediated by LPS together with a heat labile antigen. It is feasible that IL-1α is induced by more than one chlamydial antigen. LPS purified from *C. trachomatis* is shown to induce significant amounts of IL-1 from peripheral blood monocytes healthy donors (414). The same study also demonstrated that purified MOMP from *C. trachomatis* also elicited IL-1 expression, although to a much lower extent. Netea and colleagues have also reported that chlamydial antigens other than LPS stimulate cytokine production by Toll-like receptor 2 in peripheral blood (249). The possibility that another chlamydial antigen, HSP-60, is partly responsible for IL-1α seems unlikely based on previously published observations. Cytokine production is induced by chlamydial HSP-60 in various cell types, the effects of which are mediated via Toll-like receptor 4 (253,254,412). However, inhibition of Toll-like receptor 4 did not affect *C. pneumoniae* induced IL-1β production in peripheral blood mononuclear cells in the studies performed by Netea and colleagues (249).

In contrast to IL-1α induction, we showed that IL-12 expression by whole blood monocytes after *C. pneumoniae* stimulation was mediated predominantly by chlamydial LPS. Pretreatment of *C. pneumoniae* with polymixin B significantly decreased the percentage of monocytes expressing IL-12 but heat inactivation had only a minor effect. Although we have not used purified *C. pneumoniae* LPS, the specificity of polymixin B inhibitory activity against LPS (420) suggests that *C. pneumoniae* induced IL-12 expression by whole blood

monocytes is mediated via the LPS component of the bacteria. This is also in contrast to data published by Geng and colleagues who showed that although *C. pneumoniae* induced IL-12p40 (only one of the subunits of IL-12) expression, heat inactivation of bacteria before stimulation resulted in a considerable reduction in expression (248). Their data suggests that a heat labile chlamydial antigen contributes to the induction of IL-12 expression. By measuring cytokines released into cell supernatant, the observed level of expression may also have been influenced by endogenous IL-10 expression, the concentration of which was measured to be almost three fold higher than IL-12. As mentioned earlier, the current studies remove the influence of endogenous cytokine production and may provide a closer representation of direct modulation of cytokine production by *C. pneumoniae*.

E. coli LPS is known to increase IL-12 expression from peripheral blood monocytes (220,289). The predominant receptor for LPS on monocytes is the cell surface marker CD14. This receptor also aids the binding of LPS to Toll-like receptor 4, a pattern recognition receptor (424). Currently there are no reports investigating the role of Toll-like receptor 4 in IL-12 expression by *C. pneumoniae* stimulated human monocytes. However, murine bone marrow derived dendritic cells increase IL-12 expression after *C. pneumoniae* stimulation and is suggested that chlamydial endotoxin does not stimulate the cells via Toll-like receptor 4 but may activate the cells via an alternative mechanism (425). Another study utilising murine macrophages suggests that maximal LPS induced IL-12 requires not only CD14 and Toll-like receptor 4 but also another cell surface receptor, CD18/CD11b, a member of the integrin family (424). The conflicting evidence of the involvement of Toll-like receptors in LPS induced IL-12 expression may simply be a cell specific response. There are also differences between chlamydial LPS molecules in terms of potency compared to LPS derived from other bacteria (40,41). In addition, the ability of Chlamydiae to modulate the expression of surface molecules involved in LPS stimulation such as CD14 or the CD18/CD11b complex, may be a contributing factor in the pattern of cytokine response observed. Infection of monocytic cell

lines with *C. pneumoniae* are shown to increase TNF-α, IL-1β and IL-6 as well as upregulation cell surface CD14 expression (215). Polymorphisms within the CD14 promoter are also noted to regulate the density of CD14 on monocytes and has recently shown to be associated with *C. pneumoniae* infection (426).

IL-12 is composed of two subunits, p35, which is ubiquitously expressed, and p40 the promoter of which contains a number of potential transcription factor binding sites, including NFκB (427). *C. trachomatis* LPS is shown to increase nuclear translocation of NFκB in Chinese hamster ovary cells transfected with the LPS receptor CD14 (41). NFκB is also shown to regulate IL-12 production by murine macrophages that have been stimulated by LPS (427). Induction of NFκB after stimulation of whole blood monocytes by *C. pneumoniae* may play a role in the initial induction of IL-12 but is not maintained at higher concentrations of *C. pneumoniae*. NFκB activity is decreased by the inhibitory cytokine IL-10 (225) but without this influence in the current methodology (due to a Golgi block, Brefeldin A), the decreased IL-12 and possibility of decreased transcription factor activity cannot be explained by endogenous IL-10 expression. It is possible that transcription factors such as NFκB are degraded by bacterial proteases. It is reported that *C. trachomatis* produces a protease which degrades host cell transcription factors involved in MHC molecule expression (8,204,428). Therefore, *C. pneumoniae* may initially stimulate monocytes to increase IL-12 expression but once infection is established, may generate bacterial proteases capable of degrading host cell transcription factors.

In summary, we have shown that peripheral blood leucocytes are responsive to *C. pneumoniae* stimulation in terms of cytokine production. We have utilised flow cytometric techniques to identify monocytes and demonstrated that exposure to *C. pneumoniae* induces expression of TNF-α, IL-1α, IL-6, IL-8, IL-10 and IL-12 (**Figure 4.11**). In addition, we were able to demonstrate that *C. pneumoniae* antigens may regulate monocyte cytokine production

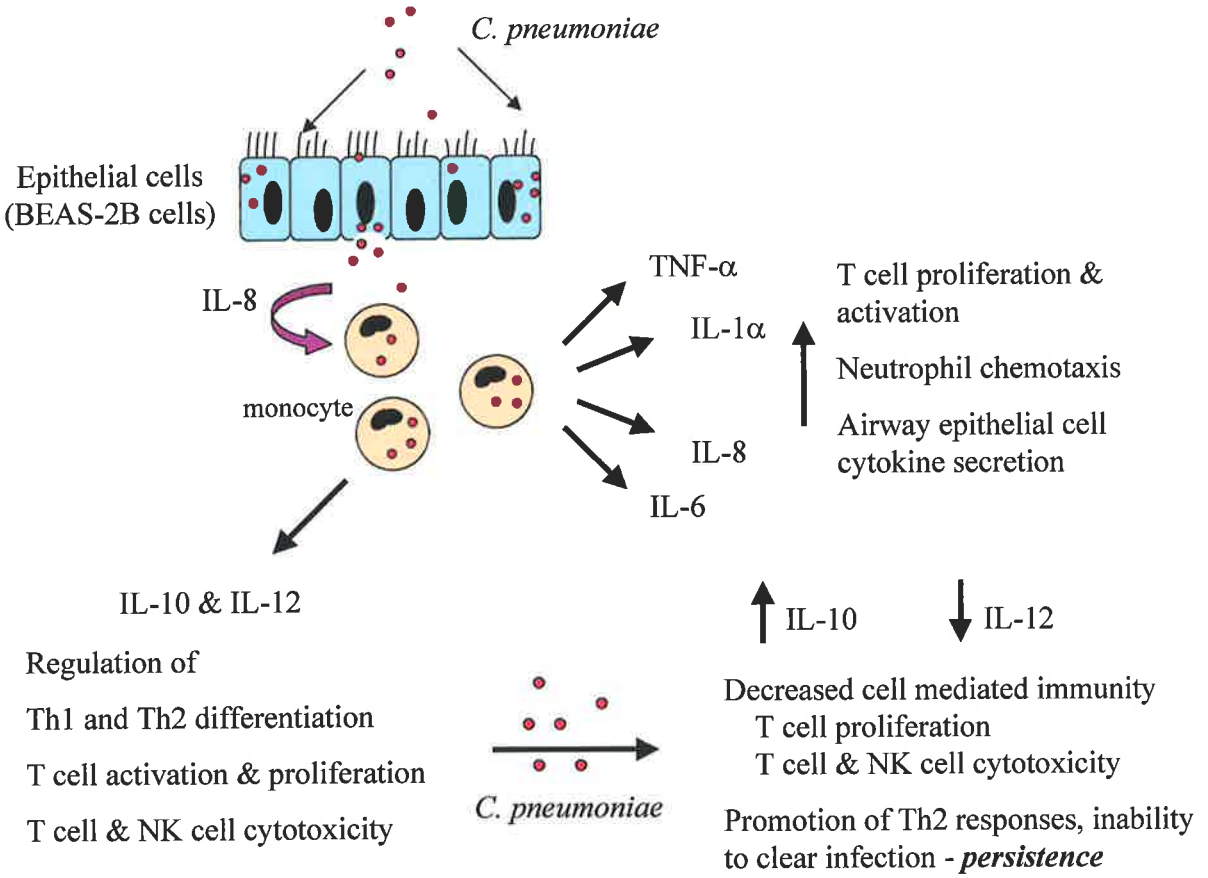


Figure 4.11: *C. pneumoniae* modulates cytokine production by peripheral blood monocytes. *C. pneumoniae* increases IL-8 production by airway epithelial cells which is chemotactic for monocytes. Monocytes respond to *C. pneumoniae* by increasing cytokine production including IL-10 and IL-12. Increased *C. pneumoniae* causes increased IL-10 (Th2) and decreased IL-12 (Th1) production by monocytes which may decrease the ability of the immune system to clear infection and promote the development of persistent infection.

via cytokine specific mechanisms. IL-1α induction by *C. pneumoniae* appeared to be mediated via more than one antigen whereas IL-12 production seemed to be predominantly induced by *C. pneumoniae* endotoxin. Interference with host cell transcriptional processes may provide a mechanism as to how *C. pneumoniae* directly modulates cytokine expression by monocytes. We also showed that lymphocytes appear not to be directly stimulated by *C. pneumoniae* and may require soluble factors secreted by accessory cells to become activated to produce cytokines.

We also observed that at higher concentrations of *C. pneumoniae*, the balance between IL-12 (Th1) and IL-10 (Th2) was modulated causing a decrease in Th1 and a corresponding increase in Th2 pattern of response. Increased IL-10 production may promote the development of persistent infection via increasing the survival of host cells. By decreasing cell mediated immunity resulting from decreased Th1 cytokine production and possibly inhibition of these responses by increased Th2 cytokine production, *C. pneumoniae* has the potential to create an environment that facilitates the development of persistent infection which may be relevant to chronic lung disease states.

CHAPTER 5

RESULTS

Surface marker expression profiles of peripheral blood leucocytes stimulated with *C. pneumoniae*

Introduction

In the previous chapter we have shown that peripheral blood leucocytes are responsive to *C. pneumoniae* stimulation in terms of cytokine production. However, the inflammatory response of host cells to infection, is not only mediated by the release of inflammatory mediators but also by cell surface. In order to establish an efficient immune response, adequate T cell activation is required. This process requires three major events; adhesion, antigen presentation and a second costimulatory signal which are all mediated in part by the expression of specific cell surface molecules (**Table 5.1**). Disruption to these processes may compromise the host defence system, possibly leading to a lack of resolution of infection and development of chronic infection. It is this persistent infection which may play a role in the inflammatory component of chronic respiratory disease such as asthma and COPD.

In cultures of peripheral blood lymphocytes obtained from healthy volunteers and asthmatics, there is no significant difference in the expression of cell surface molecules including ICAM-1, CD40, CD80, CD86 and CD28, even after infection with rhinovirus (272). However, when monocyte expression of these molecules was analysed, CD80 expression was significantly lower in the asthmatic group and these patients also expressed less CD80 in response to rhinovirus than normal individuals. Alveolar macrophages from atopic asthmatics also express reduced levels of CD80 and CD40 in comparison to control subjects (246).

SURFACE MOLECULE	LIGAND	FUNCTION
CD2 (LFA-2)	CD58 (LFA-3)	Adhesion Costimulation Enhances antigen recognition
CD3 (T cell receptor complex)	MHC molecules	Antigen recognition Signal transduction Identifies T lymphocytes
CD4	MHC Class II	Antigen recognition Identifies T helper lymphocytes
CD8	MHC Class I	Antigen recognition Identifies cytotoxic T lymphocytes
CD11a (LFA-1, α subunit of β_2 integrin)	ICAM-1 ICAM-2 ICAM-3	Adhesion
CD11b (Mac-1, α subunit of β_2 integrin, complement receptor type 3)	ICAM-1 Bacterial LPS Complement fragment iC3b	Adhesion
CD14	LPS LPS binding protein	Identifies cells of myelomonocytic lineage
CD18 (integrin β_2 subunit)		Adhesion
CD25 (α of IL-2 receptor)	IL-2	Activation marker Activation & proliferation of T cells, NK cells, macrophages, B cells
CD28	B7 family (CD80, CD86)	Costimulation of T cells Signal transduction
CD40	CD154 (CD40 ligand)	Costimulation
CD54 (ICAM-1)	LFA-1 Rhinovirus receptor	Adhesion Costimulation
CD58 (LFA-3)	CD2	Adhesion Costimulation Enhances antigen recognition
CD62L (L-selectin)	Carbohydrates such as heparan sulphate	Adhesion
CD69	Unknown	Activation marker
CD80 (B7-1)	CD28 CD152 (CTLA-4)	Costimulation of T cells Associated with Th1 responses
CD86 (B7-2)	CD28 CD152 (CTLA-4)	Costimulation of T cells Associated with Th2 responses
MHC Class I (HLA-A, -B and -C)	T cell receptor complex on $CD8^+$ T cells	Antigen presentation
MHC Class II (HLA-DR, -DQ and –DP)	T cell receptor complex on $CD4^+$ T cells	Antigen presentation

Table 5.1: Ligands and major function of cell surface molecules.
LFA = ; MHC = major histocompatibility complex; ICAM = intercellular adhesion molecule; IL = interleukin; NK = Natural Killer; CTLA = cytotoxic T lymphocyte antigen; HLA = human leucocyte antigen; Compiled from Barclay and colleagues (192).

Expression of the adhesion molecule CD11a by peripheral blood neutrophils is also similar between stable COPD patients and healthy subjects but is shown to be decreased during COPD exacerbations (367).

The expression of MHC Class II molecules between asthmatics or COPD patients and control subjects is not significantly different for a variety of cells including peripheral blood T lymphocytes, alveolar macrophages from BAL samples (246,301).

Other molecules which are indicative of activated cells such as CD25 (IL-2R), are increased on lymphocytes from bronchial biopsies of allergic asthmatics (135). In addition plasma levels of soluble IL-2R are reported to be increased in asthmatics compared to control subjects (429). Lymphocytes obtained from BAL samples from non-atopic asthmatics also show increased CD25 expression compared to normal subjects (237). However, a study comparing peripheral blood lymphocytes from healthy controls and allergic asthmatics found no significant difference in baseline cell surface expression of CD25 but it could be upregulated after viral infection (272). Similar results are reported for peripheral blood lymphocytes from COPD patients showing similar expression of CD25 compared to control patients (301).

The ability of *C. pneumoniae* to modulate surface molecule expression by cells of the immune system in lung disease has not been comprehensively studied but there is some indication that it may play an significant role. Previous work investigating the ability of *C. pneumoniae* to modulate surface marker expression on immune cells has predominantly focussed on adhesion molecules and antigen presentation. Although there are studies examining the host cell response in terms of adhesion molecule expression, they have primarily focussed on ICAM-1 expression and have been performed in cell lines. *C. pneumoniae* infection of

monocytic cell lines increased ICAM-1 expression and leads to differentiation into macrophages (252,255). There is little information published regarding freshly isolated monocytes or other leucocytes and the expression of other surface markers involved in adhesion mechanisms. Adhesion molecules not only facilitate interactions between leucocytes but also mediate adhesion with other cell types important in lung defence mechanisms such as epithelial cells and alveolar macrophages. Therefore, in order to increase our understanding of how *C. pneumoniae* modifies these interactions, it is important to investigate the expression of an assortment of adhesion molecules by a variety of cells involved in immune processes.

Detection of invading pathogens by the immune system relies on antigen presenting cells and their functional capability to process and present antigen to responsive T cells. Freshly isolated monocytes and monocytic cell lines infected with *C. pneumoniae* are reported to decrease MHC Class I expression but MHC Class II expression is not affected (252). In contrast, infection of dendritic cells or alveolar macrophages with *C. pneumoniae* causes an increase in the expression of MHC Class II molecules (130,425). These few papers demonstrate that the expression of MHC molecules may vary between cell types in response to *C. pneumoniae*.

The expression of cell surface molecules by inflammatory cells plays an important role in T cell activation. Some of these molecules are altered in chronic lung disease but the range of cells and surface molecules investigated has been relatively limited. Adequate immune responses mediated by T cell activation are necessary to maintain lung defence and clear infection. Modulation of cell surface marker expression may be a contributing factor in the development of chronic infection associated with chronic lung disease and inflammation. Preliminary data suggests that *C. pneumoniae* has the potential to alter the expression of cell surface molecules important in immune responses. However, the immune response and

interactions between inflammatory cells are highly regulated processes and are mediated by a large range of cell surface molecules. Therefore, a comprehensive investigation into the host cell-pathogen relationship in terms of *C. pneumoniae* modulation of inflammatory cell surface molecule expression is warranted.

Aims

The overall aim of the studies in this chapter was to investigate the host-pathogen relationship by describing the surface marker expression profile of leucocytes after exposure to *C. pneumoniae*.

The specific aims were:

a) To determine whether *C. pneumoniae* activates peripheral blood leucocytes cells as determined through cell surface expression of cellular activation markers.

b) To determine whether *C. pneumoniae* modulates the expression of cell surface molecules involved in adhesion processes.

c) To determine whether *C. pneumoniae* alters the expression of peripheral blood leucocyte cell surface expression of MHC molecules required for antigen presentation.

d) To determine whether *C. pneumoniae* changes the expression of cell surface molecules involved in lymphocyte costimulatory processes.

Methods

C. pneumoniae was propagated in HEp-2 cells, and harvested according to the protocol detailed in the methods chapter (Chapter 2). Uninfected cell lysate was also prepared in the same manner from uninfected HEp-2 cells. These preparations were then ready to use in stimulation experiments as described in the current chapter.

To investigate the effect of *C. pneumoniae* on the expression of cell surface molecules by whole blood mononuclear cells we utilised a stimulation protocol based on Netea and colleagues (250). Venous blood was collected into tubes containing 20 units/ml sodium heparin as an anticoagulant, from volunteers, after obtaining informed consent, who were non atopic, non smokers with no history of respiratory disease.

A single serum sample from each volunteer was also obtained to test for the presence of *C. pneumoniae* specific IgG and IgM antibodies using the MIF test (see Chapter 2) to establish a whether volunteers had previously been exposed to the bacteria.

The remaining whole blood was diluted 1:1 with serum free cell culture medium and incubated overnight with *C. pneumoniae*, a comparable concentration of uninfected cell lysate (which served as a negative control), *E. coli* LPS (100ng/ml; positive control for monocyte and neutrophil stimulation), PHA (2μg/ml; positive control for lymphocyte stimulation), or cells were incubated in media alone which served as an unstimulated control.

The expression of cell surface molecules was detected and measured using flow cytometry as described in Chapter 2 and shown in **Figure 2.13**. Whole blood monocytes were gated based on $CD14^+$ staining and side scatter characteristics (see **Figure 2.14**). Whole blood neutrophils were gated based on forward and side scatter characteristics (see **Figure 2.15**). Whole blood lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics (see **Figure**

2.16). These cells were then formatted in dot plots displaying positive staining for cell surface molecules involved in adhesion, activation, antigen presentation and costimulation processes. The relative amount of protein expressed on the cell surface could also be determined by selecting gated cells (monocytes, neutrophils or lymphocytes) and formatting these cells in histograms displaying the mean fluorescent intensity of cells staining positively for the cell surface molecule of interest (see **Figure 2.14 – 2.16**).

Results

To determine whether volunteers had previously been exposed to *C. pneumoniae*, serum from each blood sample was analysed for *C. pneumoniae* specific antibodies using the MIF test. Only one individual was identified as having negative serology to *C. pneumoniae* with no evidence of past infection (negative for IgG at 1:16 dilution) and no evidence of primary or acute infection (negative for IgM at 1:10 dilution) as described in the methods chapter. All remaining serum samples were positive for *C. pneumoniae* specific IgG antibodies analysed at a single dilution of 1:16. The criteria used in the current study to identify individuals with pre-existing antibody or evidence of past infection is in accordance with previously defined criteria (3,84,88). These studies have defined past infection as a serum sample with an IgG titre ≥1:16 and <1:512. The results obtained from the MIF test suggest that all subjects, except one, participating in this study had evidence of past infection with *C. pneumoniae*.

Does C. pneumoniae activate peripheral blood monocytes?

In order to assess whether exposure of whole blood monocytes to *C. pneumoniae* changes their state of activation, we measured the expression of surface molecules, indicative of cellular activation. Using flow cytometry we first measured the expression of CD69, a marker of early activation. Less than 20% of unstimulated monocytes expressed CD69 (**Figure 5.1**). However, stimulation with *E. coli* LPS resulted in a significant increase in the percentage of monocytes expressing CD69, up to 45%. Although stimulation with low concentrations of *C. pneumoniae* (IOL-207, $1x10^6$ ifu/ml) did not significantly alter CD69 expression, the highest concentration of *C. pneumoniae*, $1x10^7$ ifu/ml, caused an increase in the percentage of monocytes expressing CD69 up to 76%, in comparison to an appropriate amount of uninfected cell lysate.

The amount of CD69 expression per cell could also be determined by flow cytometry by measuring the mean fluorescent intensity of monocytes staining positively for CD69. In comparison to unstimulated cells, monocytes that were incubated with *E. coli* LPS overnight,

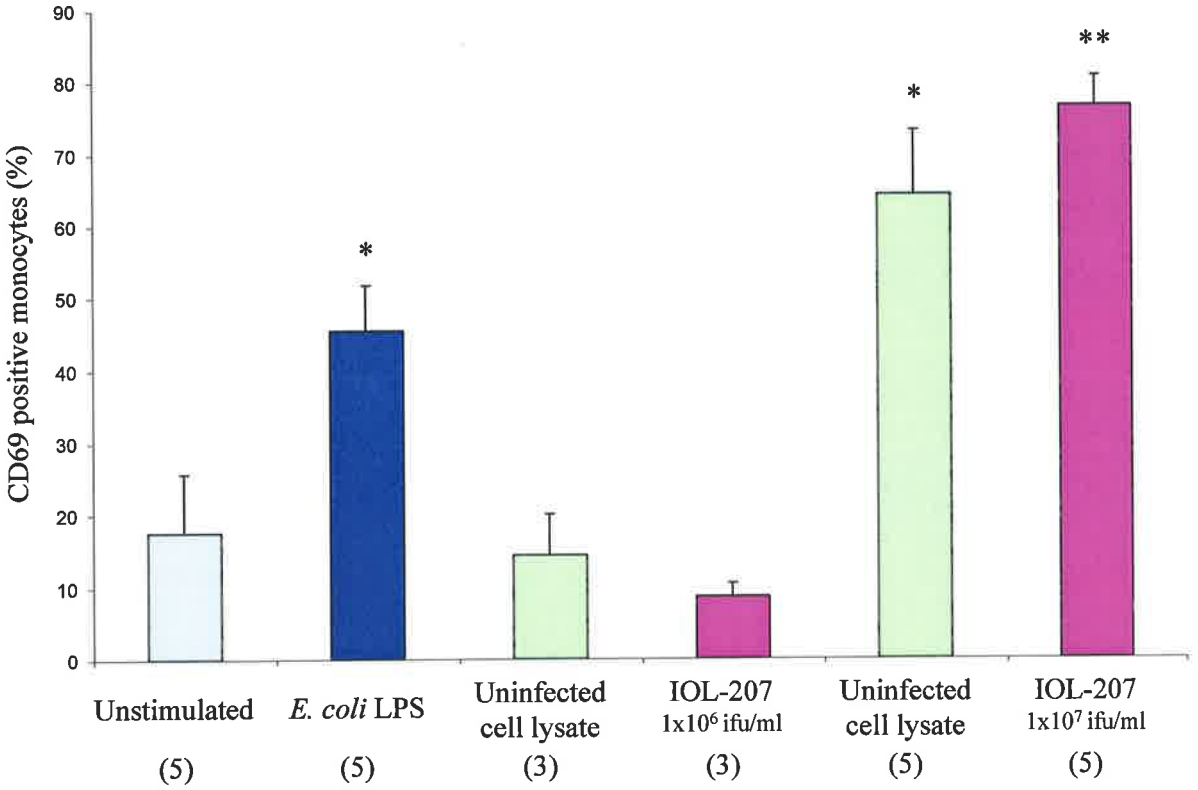


Figure 5.1: *C. pneumoniae* increases the percentage of monocytes expressing CD69 at high concentrations. Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and CD69 positive cells were detected using an anti-human CD69 PE labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

showed an increase in CD69 MFI (**Figure 5.2**). In addition, in comparison to comparable amounts of uninfected cell lysate, both concentrations of *C. pneumoniae* increased CD69 MFI on monocytes.

We also assessed the expression of a second marker of cellular activation, CD25, which is an indicator of late activation. Monocytes selected from whole blood that was incubated overnight with no additional stimulus (unstimulated) showed minimal expression of CD25 (**Figure 5.3**). The percentage of monocytes expressing CD25 was dramatically increased to over 90% after stimulation with *E. coli* LPS. Although incubation with uninfected cell lysate caused an increase in the percentage of monocytes expressing CD25 compared to uninfected cell lysate, the effect of incubating whole blood with *C. pneumoniae* was significant. Both concentrations of *C. pneumoniae* added to whole blood cultures caused the percentage of monocytes expressing CD25 to increase approximately 3 fold, in comparison to cultures incubated with uninfected cell lysate, with over 80% of cells staining positively for CD25.

When examining the MFI of monocytes expressing CD25, a similar pattern of modulation was observed. In comparison to unstimulated cells, *E. coli* LPS significantly increased the amount of CD25 expressed on the cell surface (**Figure 5.4**). A small but significant increase in MFI was observed after incubation with the lowest amount of uninfected cell lysate but the higher amount was not different to unstimulated cells. Despite this, after exposure to *C. pneumoniae*, monocytes expressing CD25 significantly increased the amount of CD25 protein measured on the cell surface.

Does C. pneumoniae activate whole blood neutrophils?

We also assessed the expression of CD69 and CD25 on whole blood neutrophils. Similar to the results obtained with monocytes, approximately 10% of resting neutrophils constitutively expressed CD69 and this was significantly increased to 64% after incubation with *E. coli* LPS

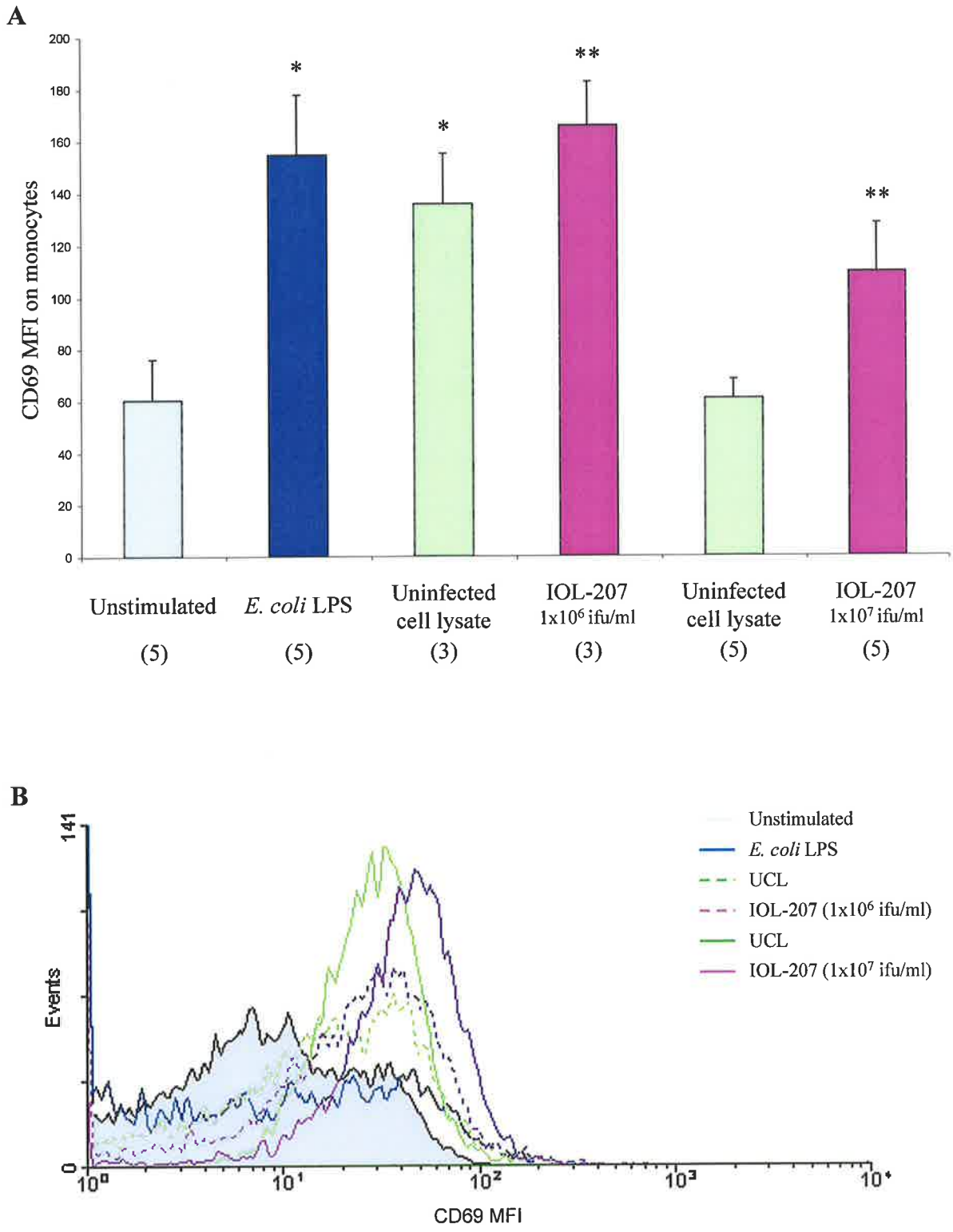


Figure 5.2: *C. pneumoniae* increases the CD69 MFI on monocytes.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and CD69 MFI was determined using an anti-human CD69 PE labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of monocytes displaying increased CD69 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

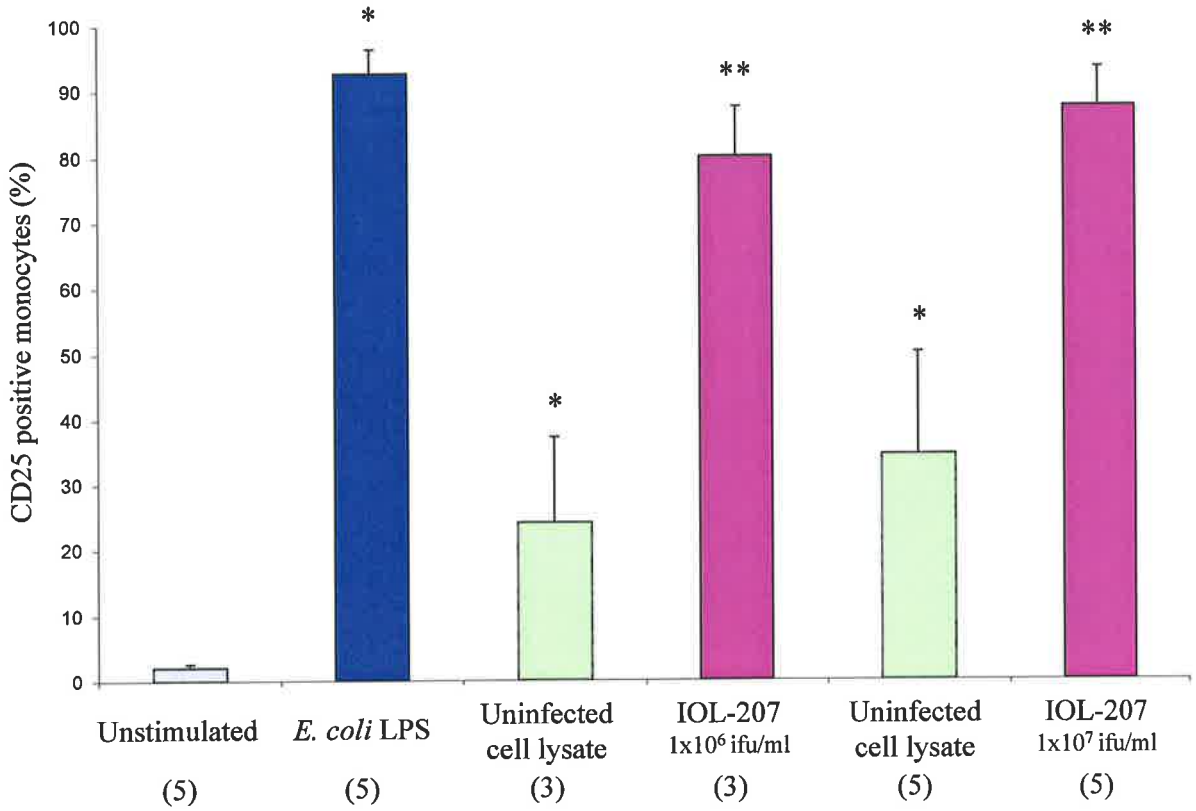


Figure 5.3: *C. pneumoniae* increases the percentage of monocytes expressing CD25.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and CD25 positive cells were detected using an anti-human CD25 FITC labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

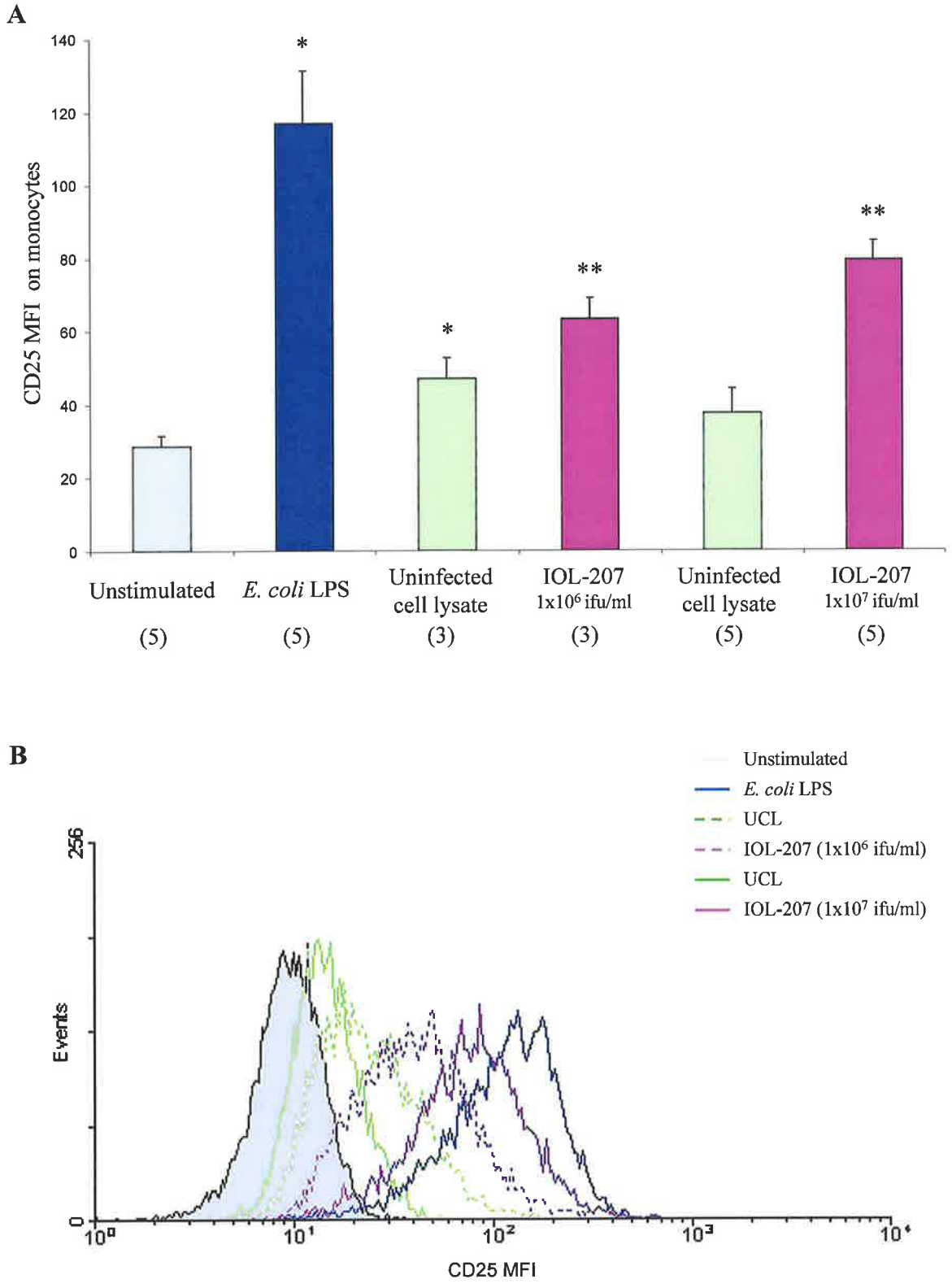


Figure 5.4: *C. pneumoniae* increases CD25 MFI on whole blood monocytes.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^{+}$ antibody staining by flow cytometry and CD25 MFI was determined using an anti-human CD25 FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of monocytes displaying increased CD25 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

(**Figure 5.5**). The lowest concentration of uninfected cell lysate significantly induced CD69 expression on 25% of neutrophils in comparison to unstimulated cells. After exposure to *C. pneumoniae*, 58% of neutrophils expressed CD69 which was significantly increased compared to cells incubated with uninfected cell lysate. The highest concentration of *C. pneumoniae*, $1x10^7$ ifu/ml caused virtually all neutrophils to express CD69 (95%).

We also analysed the MFI of neutrophils expressing CD69 and observed a significant increase of CD69 MFI on neutrophils after stimulation with *E. coli* LPS (**Table 5.2**). The trends seen with increased CD69 expression by neutrophils after stimulation with *C. pneumoniae* were less obvious in the MFI data. However, a significant increase in neutrophil CD69 MFI was noted at the highest concentration of *C. pneumoniae*, compared to cells incubated with uninfected cell lysate.

MFI of neutrophils expressing CD69						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	39.61	63.82	51.64	54.03	42.77	46.53
2	39.95	54.36	52.53	45.63	37.17	31.19
3	32.59	47.53	32.29	41.19	32.96	46.38
4	31.34	57.42	nd	nd	27.46	43.83
5	52.49	35.46	nd	nd	33.70	43.02
Mean	39.20	51.72*	45.49	46.95	34.81	42.19**
SEM	3.76	4.84	6.60	3.76	2.53	2.84

Table 5.2: Effect of *C. pneumoniae* on MFI of neutrophils expressing CD69.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were selected based on forward and side scatter characteristics. CD69 expression was detected using an anti-human PE labelled monoclonal antibody and detected using flow cytometry. Data represents the mean percentage of neutrophils expressing CD69 from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to UCL, mixed ANOVA.

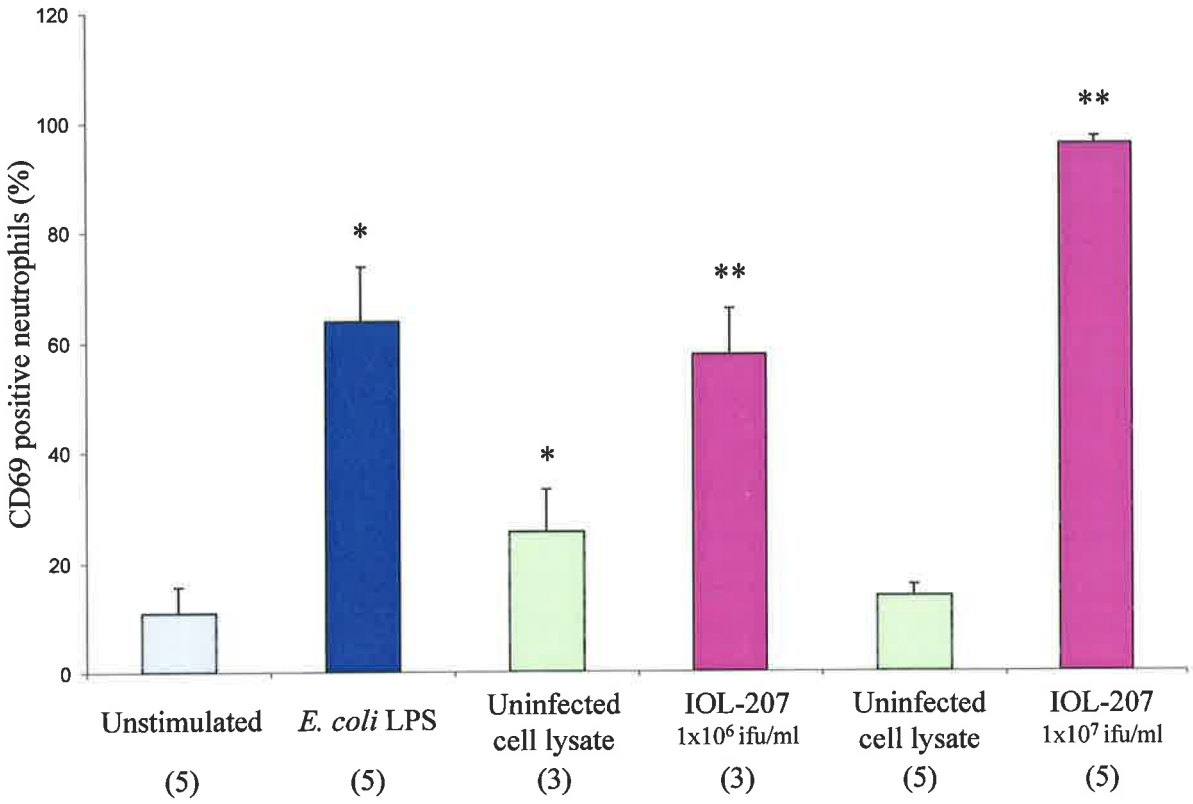


Figure 5.5: *C. pneumoniae* increases the percentage of neutrophils expressing CD69.
Whole blood neutrophils were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD69 positive cells were detected using an anti-human CD69 PE labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

We also investigated cell surface expression of CD25 on neutrophils but did not detect expression on unstimulated cells. In addition the expression of CD25 was not induced under any of the conditions tested.

Does C. pneumoniae activate whole blood lymphocytes?

After assessing the activation state of monocytes and neutrophils we also investigated whether *C. pneumoniae* altered the expression of activation markers on lymphocytes. The expression of CD69 on unstimulated lymphocytes was approximately 5% and was significantly increased to 50% after exposure to PHA (**Figure 5.6**). The percentage of lymphocytes expressing CD69 after incubation with uninfected cell lysate was not significantly different to unstimulated cells. However, we found that *C. pneumoniae* significantly induced CD69 expression on 40% of lymphocytes as compared to uninfected cell lysate. In addition, the MFI of lymphocytes expressing CD69 also reflected these results with *C. pneumoniae* causing a 2.5 fold increase in the amount of CD69 protein expressed on the cell surface (**Figure 5.7**).

We also investigated the effect of *C. pneumoniae* stimulation of lymphocytes on their expression of CD25. As can be seen in **Figure 5.8**, CD25 expression was detected on unstimulated lymphocytes and a small but significant increase in the percentage of lymphocytes expressing CD25 was seen after stimulation with PHA (33%). However, the percentage of lymphocytes expressing CD25 was not significantly altered after exposure to uninfected cell lysate or *C. pneumoniae*. PHA significantly increased the amount of CD25 expression per cell, as determined by CD25 MFI (**Figure 5.9**). Uninfected cell lysate did not change CD25 MFI of lymphocytes but overnight exposure to *C. pneumoniae* caused a significant increase in the amount of CD25 expression on lymphocytes compared to cells exposed to uninfected cell lysate.

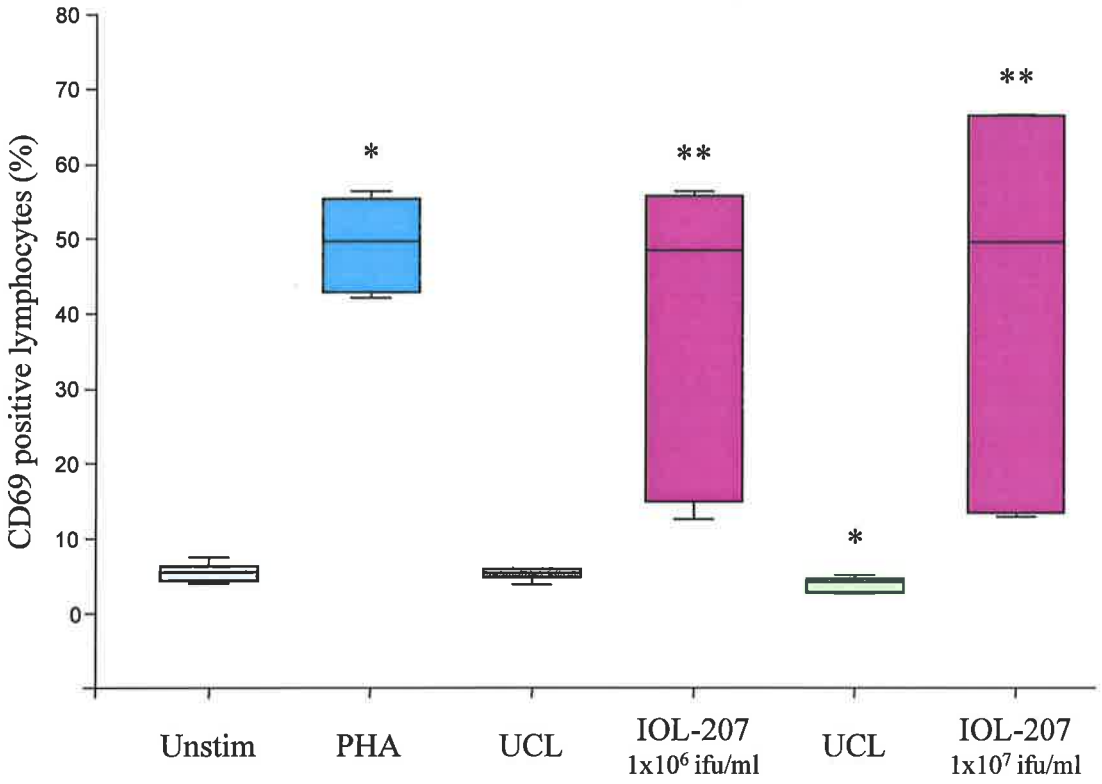


Figure 5.6: *C. pneumoniae* increases the percentage of lymphocyte expressing CD69.
Whole blood lymphocytes were incubated overnight with PHA (2μg/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics by flow cytometry and CD69 positive cells were detected using an anti-human CD69 PE labelled monoclonal antibody. Boxes represent the interquartile range of 3 separate experiments performed in triplicate. Whiskers represent the highest and lowest values, with the line across the box representing the median. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA. The reciprocal of the raw data was calculated before applying the mixed ANOVA model for analysis.

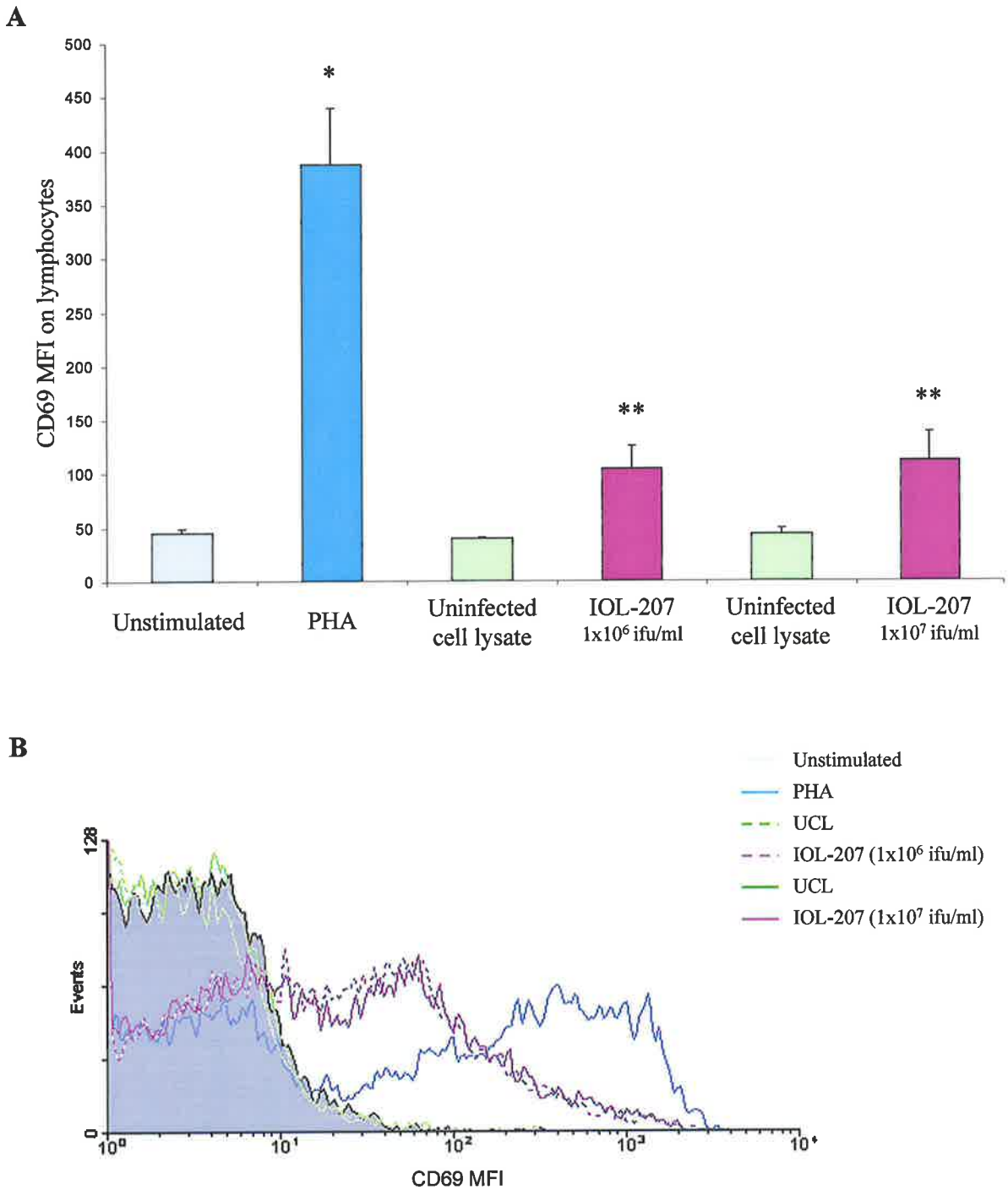


Figure 5.7: *C. pneumoniae* increases MFI of lymphocytes expressing CD69.
Whole blood lymphocytes were incubated overnight with PHA (2µg/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics by flow cytometry and CD69 MFI was determined using an anti-human CD69 PE labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of 3 separate experiments performed in triplicate. **B)** Histogram is representative of lymphocytes displaying increased CD69 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

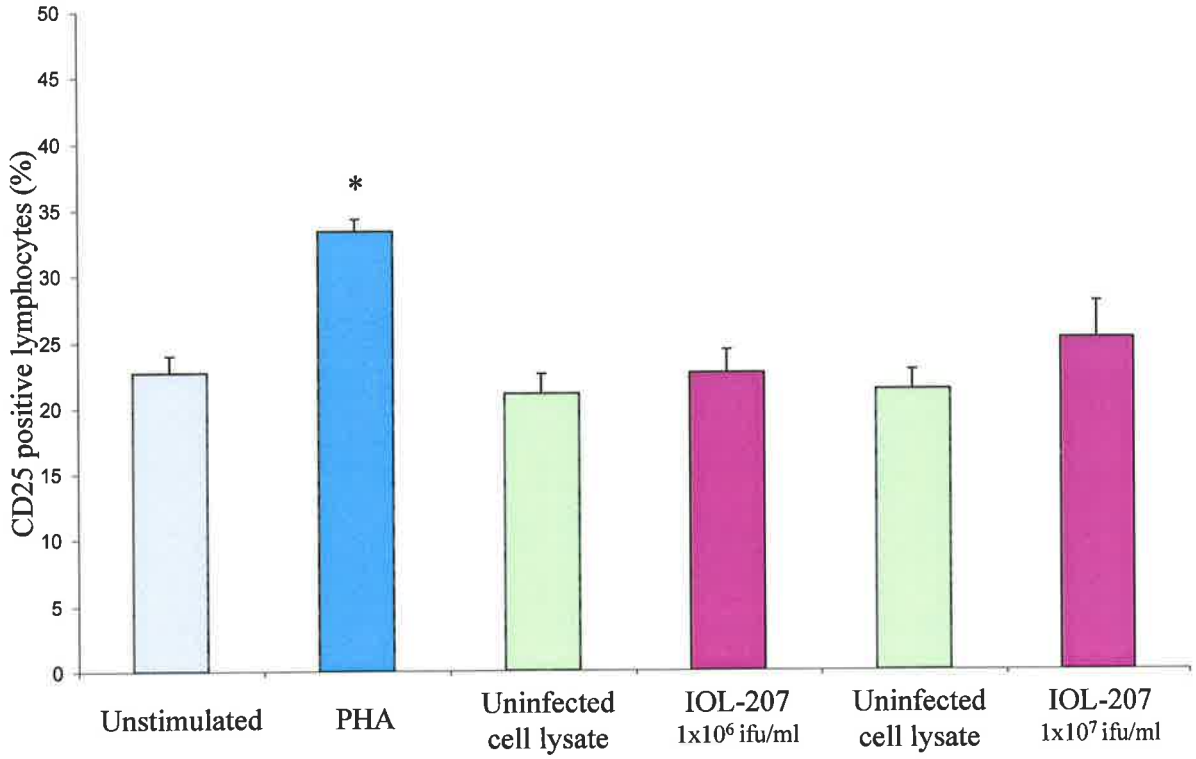


Figure 5.8: *C. pneumoniae* does not effect the percentage of lymphocyte expressing CD25.
Whole blood lymphocytes were incubated overnight with PHA (2µg/ml), uninfected cell lysate (UCL) or *C pneumoniae* (IOL-207). Lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics by flow cytometry and CD25 positive cells were detected using an anti-human CD25 FITC labelled monoclonal antibody. Bars represent the mean ± SEM of 3 separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA.

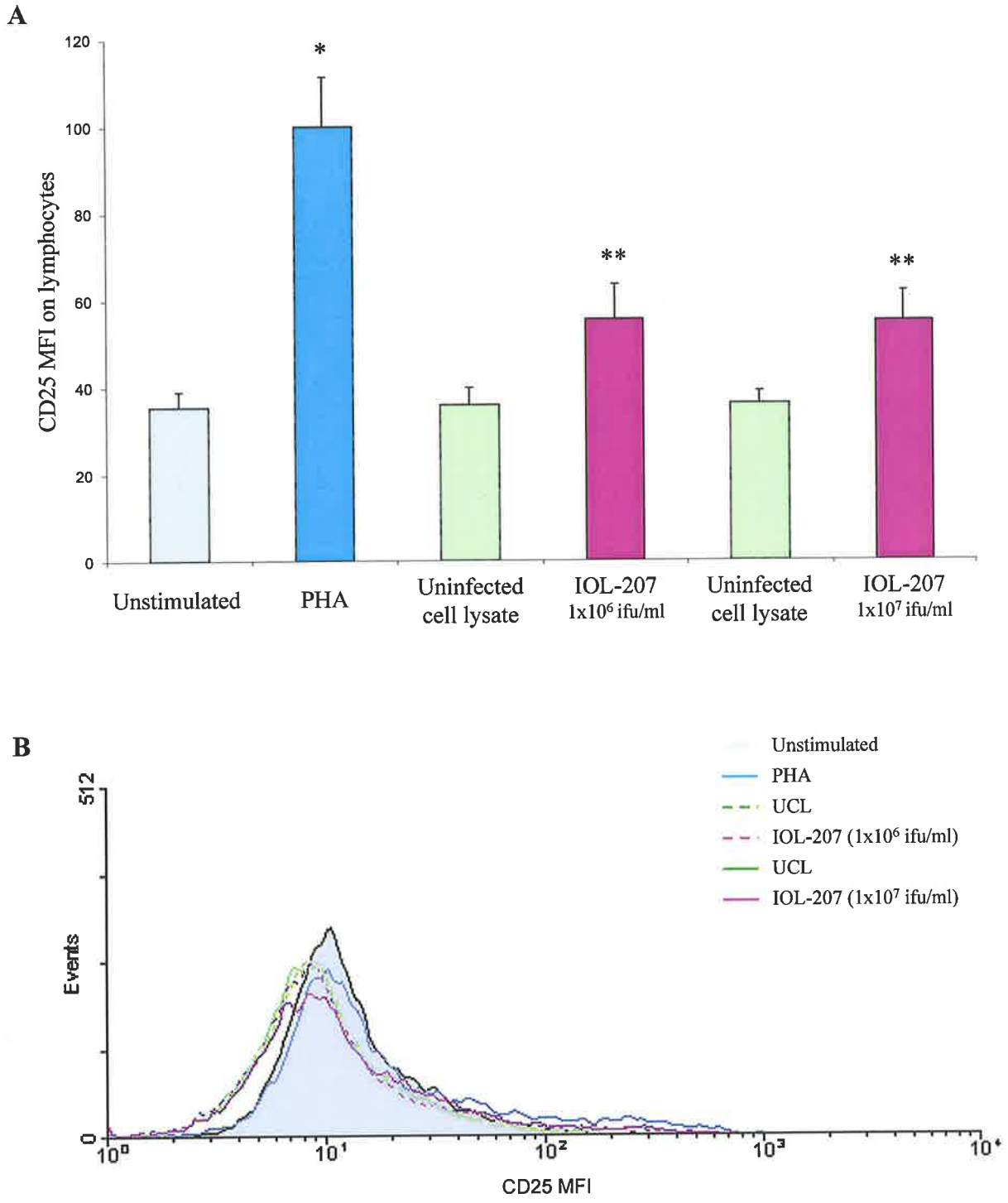


Figure 5.9: *C. pneumoniae* increases CD25 expression by lymphocytes.
Whole blood lymphocytes were incubated overnight with PHA (2μg/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics by flow cytometry and CD25 MFI was determined using an anti-human CD25 FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of 3 separate experiments performed in triplicate. **B)** Histogram is representative of lymphocytes displaying increased CD25 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

Surface expression of CD69 and CD25 gives an indication of whether a cell is in an activated state or not. We have investigated whether *C. pneumoniae* alters the expression of these molecules on leucocytes from whole blood and are summarised in **Table 5.3**.

Summary of activation marker expression on leucocytes after *C. pneumoniae* exposure			
Surface molecule	**Monocyte**	**Neutrophil**	**Lymphocyte**
CD69	↑	↑	↑
CD25	↑	nd	↑

Table 5.3: *C. pneumoniae* increases the expression of activation molecules on leucocytes.
nd, not detected; ↑, increased.

Does C. pneumoniae modulate monocyte expression of adhesion molecules?
One of the first requirements for adequate T cell activation is adhesion between antigen presenting cells and T cells. This interaction is governed by the expression of adhesion molecules and our aim was to determine whether *C. pneumoniae* has the capacity to alter monocyte expression of these molecules. As can be seen in **Table 5.4** practically the entire population of monocytes, whether unstimulated or stimulated, expressed ICAM-1, as detected by flow cytometry.

Percentage of monocytes expressing ICAM-1						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	95.49	100.00	98.18	99.73	99.37	97.79
2	47.00	99.89	99.47	99.77	87.95	100.00
3	78.79	99.97	99.53	99.98	99.93	98.59
4	96.14	100.00	nd	nd	99.95	99.69
5	89.56	100.00	nd	nd	100.00	100.00
Mean	81.39	99.97*	99.06*	99.83	97.44*	99.21
SEM	9.15	0.02	0.44	0.08	2.37	0.44

Table 5.4: *C. pneumoniae* does not effect the percentage of monocytes expressing ICAM-1.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on CD14$^+$ staining and side scatter characteristics. ICAM-1 expression was detected using an anti-human PE labelled monoclonal ICAM-1 antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing ICAM-1 from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$ compared to unstimulated, mixed ANOVA. Raw data was cubed before applying the mixed ANOVA model for analysis.

However, analysis of the mean fluorescent intensity of monocytes expressing ICAM-1 showed that in comparison to unstimulated cells, *E. coli* LPS significantly upregulated ICAM-1 MFI (**Figure 5.10**). Furthermore, in comparison to appropriate amounts of uninfected cell lysate, *C. pneumoniae* significantly increased the MFI of monocytes expressing ICAM-1 approximately 1.5 fold. As there was no significant difference in the MFI of monocytes expressing ICAM-1 after incubation with different amounts of uninfected cell lysate, a comparison between the two concentrations of *C. pneumoniae* could be made, but the MFI was not different between the two concentrations.

Monocyte expression of a second molecule, CD58, primarily involved in adhesion mechanisms, was also evaluated after stimulation of whole blood with *C. pneumoniae*. As shown in **Table 5.5**, similar to the results obtained with ICAM-1 expression, CD58 was constitutively expressed by the majority of unstimulated cells and remained unchanged after any of the cell treatments examined.

Percentage of monocytes expressing CD58						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	99.97	99.99	99.98	99.97	100.00	94.86
2	99.99	99.90	99.73	97.94	99.98	99.94
3	99.97	99.92	99.78	99.95	99.98	96.83
4	99.99	99.98	nd	nd	99.99	100.00
5	99.99	99.97	nd	nd	99.98	99.29
Mean	99.98	99.95	99.83	99.29	99.99	98.18
SEM	0.01	0.02	0.08	0.67	0.00	1.01

Table 5.5: *C. pneumoniae* does not effect the percentage of monocytes expressing CD58.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. CD58 expression was detected using an anti-human FITC labelled monoclonal CD58 antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing CD58 from 5 individual subjects, performed in triplicate. nd: not done.

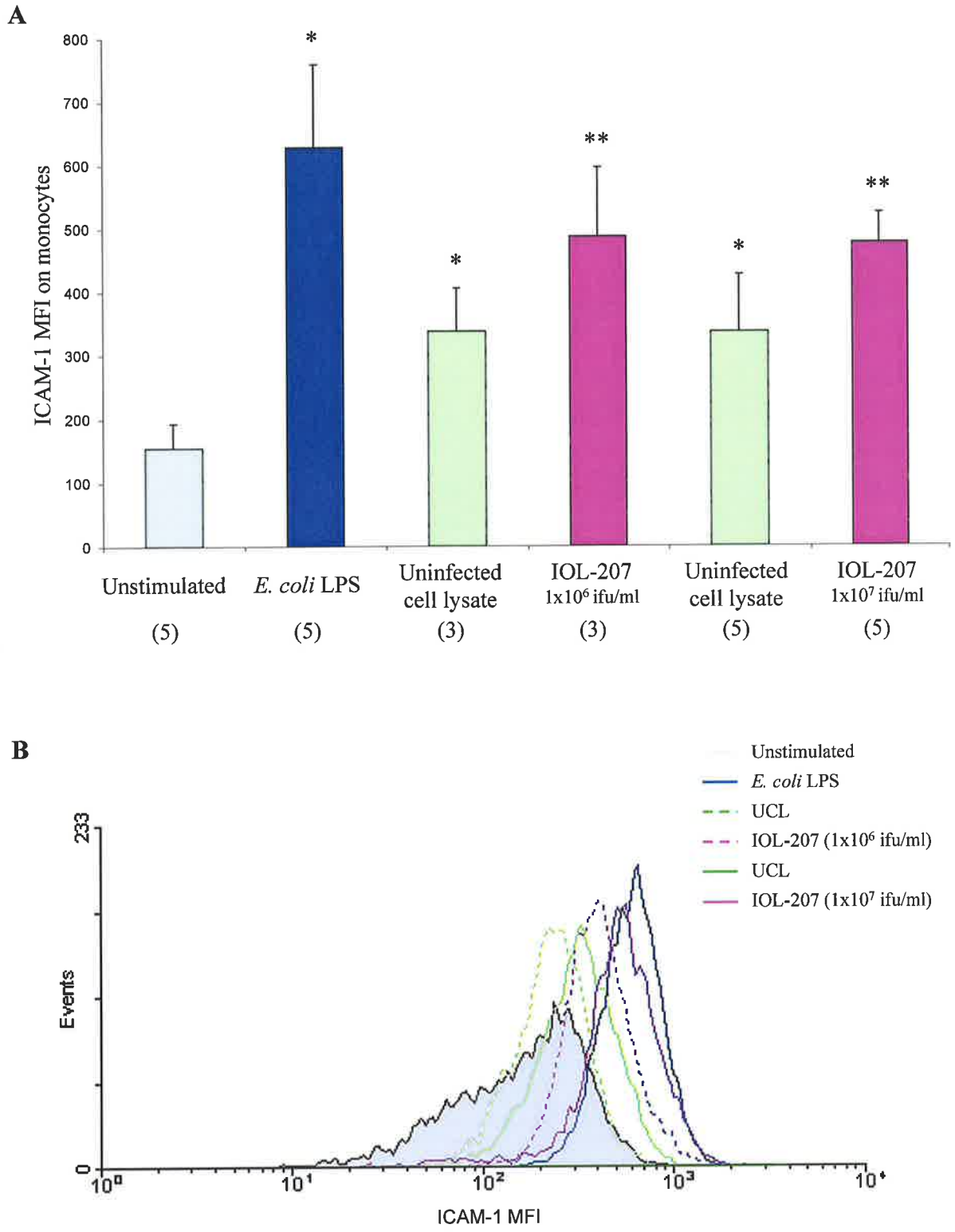


Figure 5.10: *C. pneumoniae* increases ICAM-1 expression by monocytes.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^{+}$ antibody staining by flow cytometry and ICAM-1 MFI was determined using an anti-human ICAM-1 PE labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of monocytes displaying increased ICAM-1 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

We then analysed the MFI of monocytes expressing CD58 (**Table 5.6**). We observed that there were no significant changes in the MFI of monocytes expressing CD58 after overnight incubation with *C. pneumoniae* or any other stimulus.

Mean fluorescent intensity of monocytes expressing CD58						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	91.49	83.13	81.81	69.76	90.57	70.79
2	81.86	85.08	76.09	60.57	83.32	76.52
3	82.55	76.06	87.81	79.44	80.91	114.67
4	83.14	85.36	nd	nd	78.41	79.49
5	77.73	92.12	nd	nd	91.76	66.42
Mean	83.35	84.35	81.90	69.93	84.99	81.58
SEM	2.24	2.57	3.39	5.45	2.64	8.58

Table 5.6: *C. pneumoniae* does not effect the MFI of monocytes expressing CD58.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ staining by flow cytometry and CD58 MFI was determined using an anti-human FITC labelled monoclonal CD58 antibody. Data represents the mean MFI of monocytes expressing CD58 from 5 individual subjects, performed in triplicate. nd: not done.

Another molecule involved in monocyte adhesion processes is CD11a. Similar to the results obtained with ICAM-1 and CD58 expression, the percentage of monocytes expressing CD11a on unstimulated cells was 100% and remained unchanged after exposure to any of the stimuli tested (**Table 5.7**).

Percentage of monocytes expressing CD11a						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	99.99	99.99	99.96	99.93	100.00	94.88
2	100.00	99.98	99.52	96.49	100.00	99.78
3	99.99	99.86	99.96	99.91	99.95	91.75
4	99.99	98.54	nd	nd	99.66	83.03
5	99.97	99.92	nd	nd	99.96	98.07
Mean	99.99	99.66	99.81	98.78	99.92	93.50
SEM	0.00	0.28	0.15	1.14	0.06	2.96

Table 5.7: *C. pneumoniae* does not effect the percentage of monocytes expressing CD11a.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. CD11a expression was detected using an anti-human FITC labelled monoclonal CD11a antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing CD11a from 5 individual subjects, performed in triplicate. nd: not done.

Analysis of CD11a MFI showed that in comparison to unstimulated cells, *E. coli* LPS did not change the amount of CD11a being expressed on the cell surface of monocytes (**Figure 5.11**). However, in contrast to these results, stimulation of whole blood cultures with *C. pneumoniae* induced a significant decrease in the MFI of monocytes expressing CD11a in comparison to cells incubated with uninfected cell lysate. In addition, as there was a statistical difference between unstimulated cells and cells exposed to uninfected cell lysate, but no statistically significant difference found between the amounts of uninfected cell lysate, a comparison could be made between the two concentrations of *C. pneumoniae*. We found that the decrease in CD11a MFI caused by incubation with the highest concentration of *C. pneumoniae* ($1x10^7$ ifu/ml) was also significantly less than that observed with the *C. pneumoniae* preparation containing $1x10^6$ ifu/ml.

Does C. pneumoniae modulate neutrophil expression of adhesion molecules?
Neutrophils also express a number of adhesion molecules which govern the interactions with other leucocytes and cells such as airway epithelium. We therefore, also investigated neutrophil expression of adhesion molecules after exposure to *C. pneumoniae*. Unlike

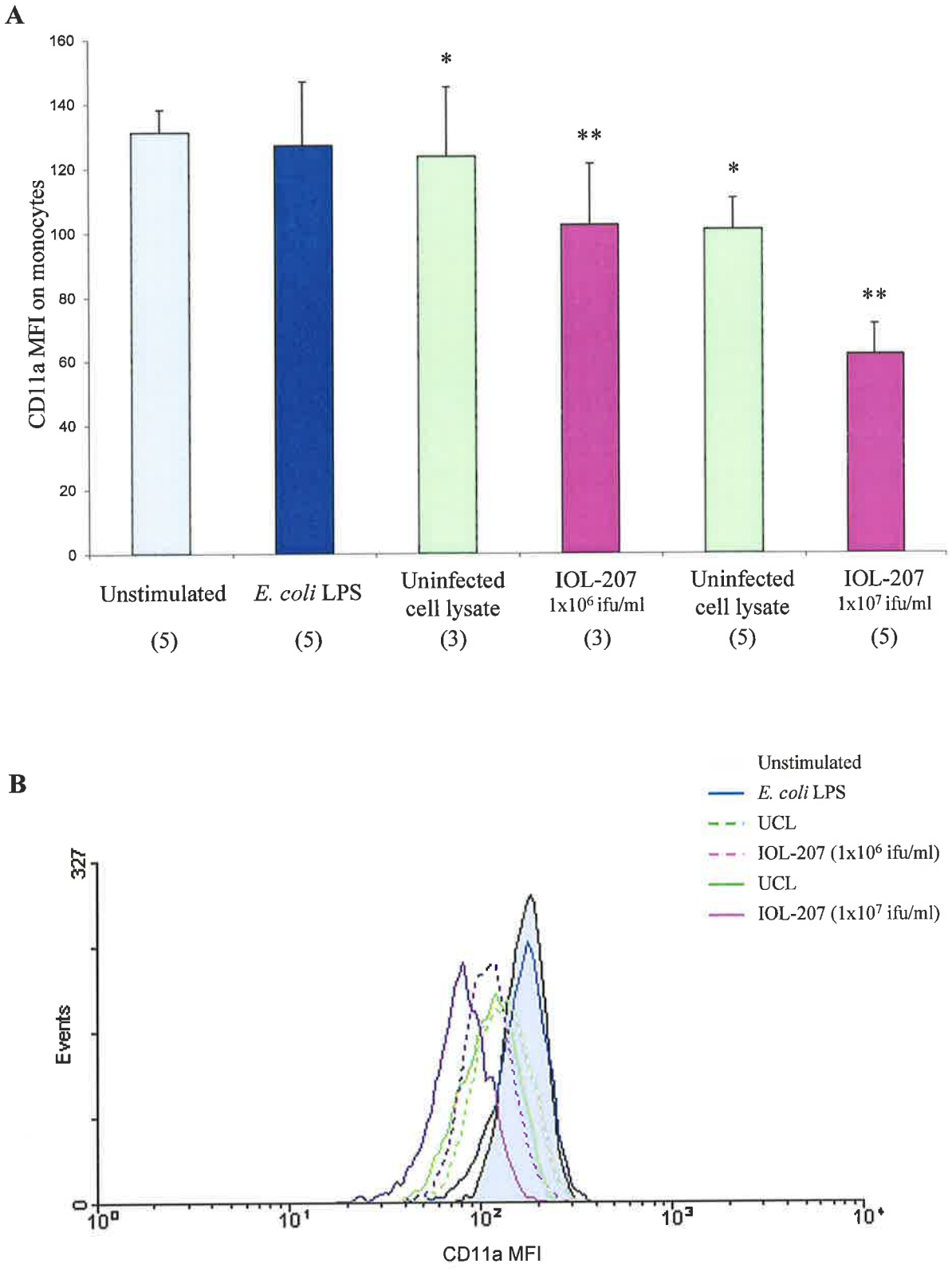


Figure 5.11: *C. pneumoniae* decreases monocyte CD11a MFI.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and CD11a MFI was determined using an anti-human CD11a FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of monocytes displaying decreased CD11a MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

monocytes, the majority of neutrophils do not constitutively express ICAM-1 with less than 20% exhibiting positive staining (**Figure 5.12**). However, after overnight stimulation with *E. coli* LPS, ICAM-1 expression was induced on the majority of neutrophils. There were contrasting results with exposure of neutrophils to uninfected cell lysate. The lowest volume used resulted in a significant increase in the percentage of neutrophils expressing ICAM-1 whereas the highest amount did not significantly change the percentage of cells staining positive for ICAM-1 from cells that were unstimulated. Nevertheless, a consistent increase in neutrophils expressing ICAM-1 was seen when whole blood was incubated overnight with *C. pneumoniae* (up to 97%) compared to the appropriate amount of uninfected cell lysate. Similar results were seen when analysing the MFI of neutrophils expressing ICAM-1. Compared to Unstimulated cells, *E. coli* LPS stimulation caused a significant shift in ICAM-1 MFI demonstrating increased expression (**Figure 5.13**). Uninfected cell lysate also caused a small but significant increase in ICAM-1 MFI at the lower concentration but the higher amount was similar to unstimulated cells. Consistent with the percentage of neutrophils expressing ICAM-1, the amount of ICAM-1 protein (as indicated by the MFI) expressed on the neutrophil surface was also increased after incubation with *C. pneumoniae*, compared to uninfected cell lysate.

Neutrophils also express CD58 and we determined that CD58 was constitutively expressed on all unstimulated neutrophils (**Table 5.8**). This percentage was unchanged under all treatment conditions tested.

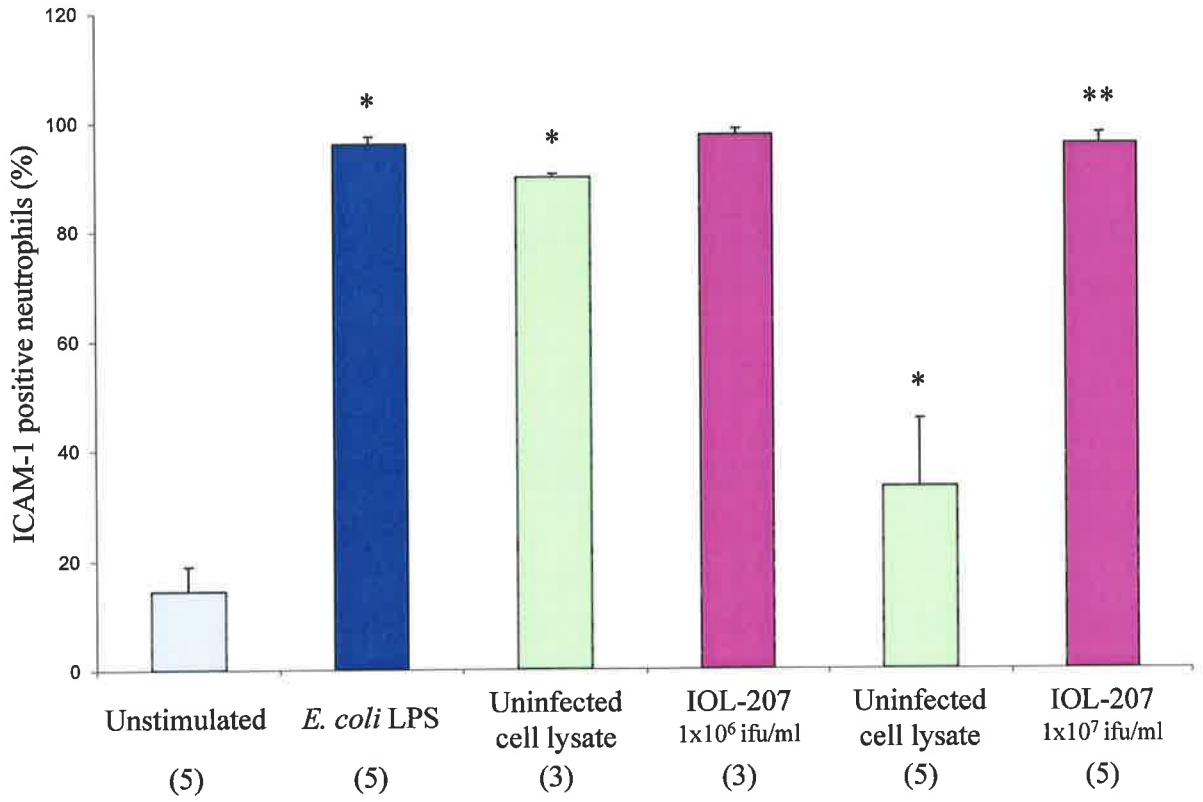


Figure 5.12: *C. pneumoniae* increases the percentage of neutrophils expressing ICAM-1.
Whole blood neutrophils were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and ICAM-1 positive cells were detected using an anti-human ICAM-1 PE labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

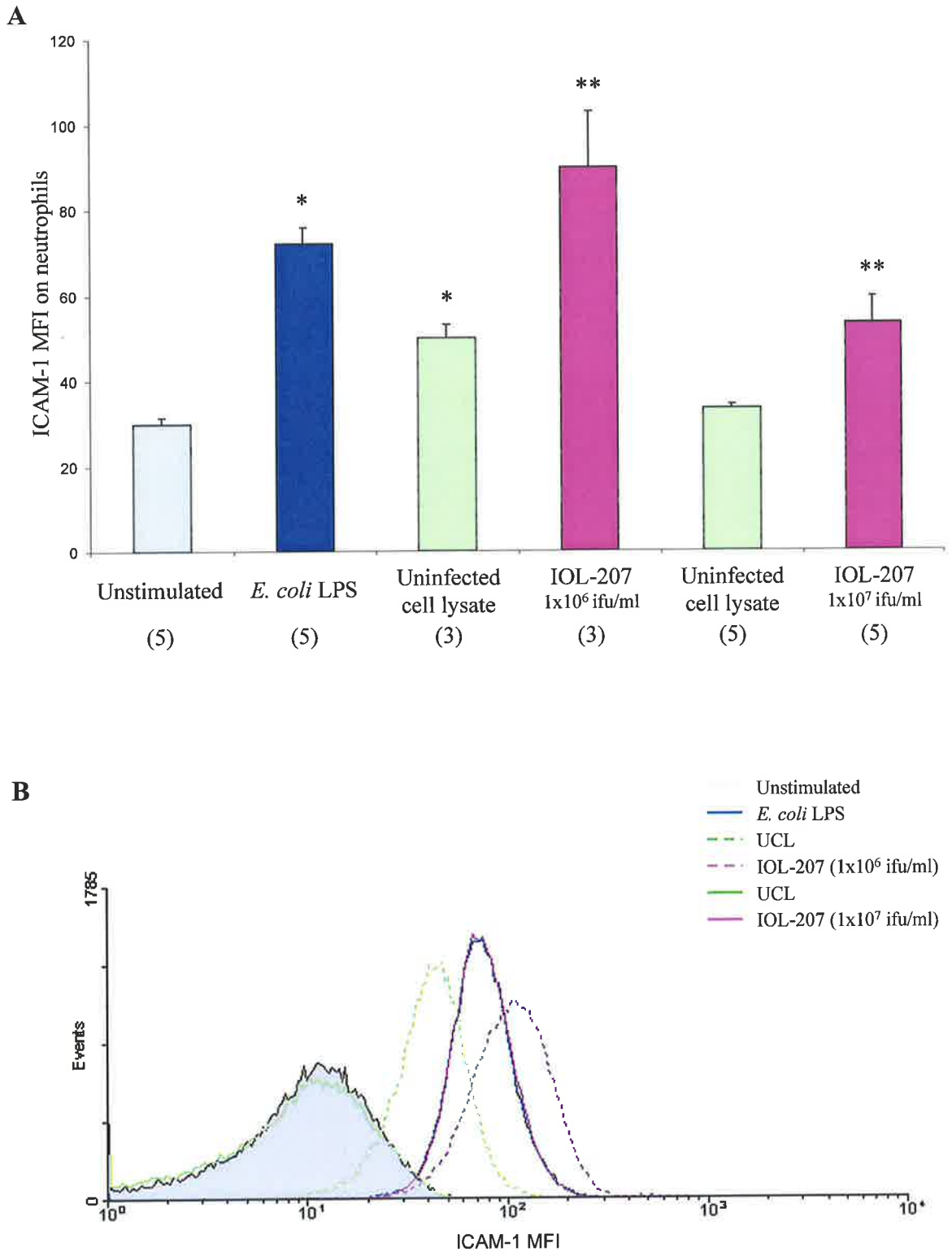


Figure 5.13: *C. pneumoniae* increases ICAM-1 expression by neutrophils.
Whole blood neutrophils were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and ICAM-1 MFI was determined using an anti-human ICAM-1 PE labelled monoclonal antibody. **A**) Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B**) Histogram is representative of neutrophils displaying increased ICAM-1 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

Percentage of neutrophils expressing CD58						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	98.27	95.05	89.01	88.17	98.04	98.18
2	98.17	98.60	96.76	83.80	98.95	99.91
3	98.95	97.60	97.06	74.70	92.00	89.21
4	99.93	98.29	nd	nd	99.83	98.98
5	97.41	93.11	nd	nd	97.84	92.80
Mean	98.55	96.53	94.28*	82.22**	97.33	95.82
SEM	0.42	1.06	2.63	3.97	1.38	2.06

Table 5.8: *C. pneumoniae* does not effect the percentage of neutrophils expressing CD58.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were selected based on forward and side scatter characteristics. CD58 expression was detected using an anti-human FITC labelled monoclonal CD58 antibody and detected using flow cytometry. Data represents the mean percentage of neutrophils expressing CD58 from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of (UCL), mixed ANOVA. Raw data was squared before applying the mixed ANOVA model for analysis.

It was then necessary to assess the effect of *C. pneumoniae* stimulation on neutrophil CD58 expression by analysing the MFI. As can be seen in **Figure 5.14**, exposure of whole blood neutrophils to *E. coli* LPS significantly decreased the MFI of neutrophils expressing CD58. Uninfected cell lysate had no effect on CD58 MFI but stimulation with *C. pneumoniae* caused a significant decrease similar to that observed after stimulation with *E. coli* LPS.

Similar to neutrophil CD58 expression, CD11a was also expressed constitutively on unstimulated neutrophils (**Table 5.9**). After exposure to *E. coli* LPS, there was a small decrease in the percentage of neutrophils expressing CD11a. The percentage of neutrophils expressing CD11a was significantly decreased after overnight incubation with low amounts of uninfected cell lysate to approximately 80% which was further decreased after exposure to *C. pneumoniae* ($1x10^6$ ifu/ml). The higher amount of uninfected cell lysate did not significantly change CD11a expression by neutrophils (compared to unstimulated cells) and expression was retained after exposure to *C. pneumoniae* ($1x10^7$ ifu/ml).

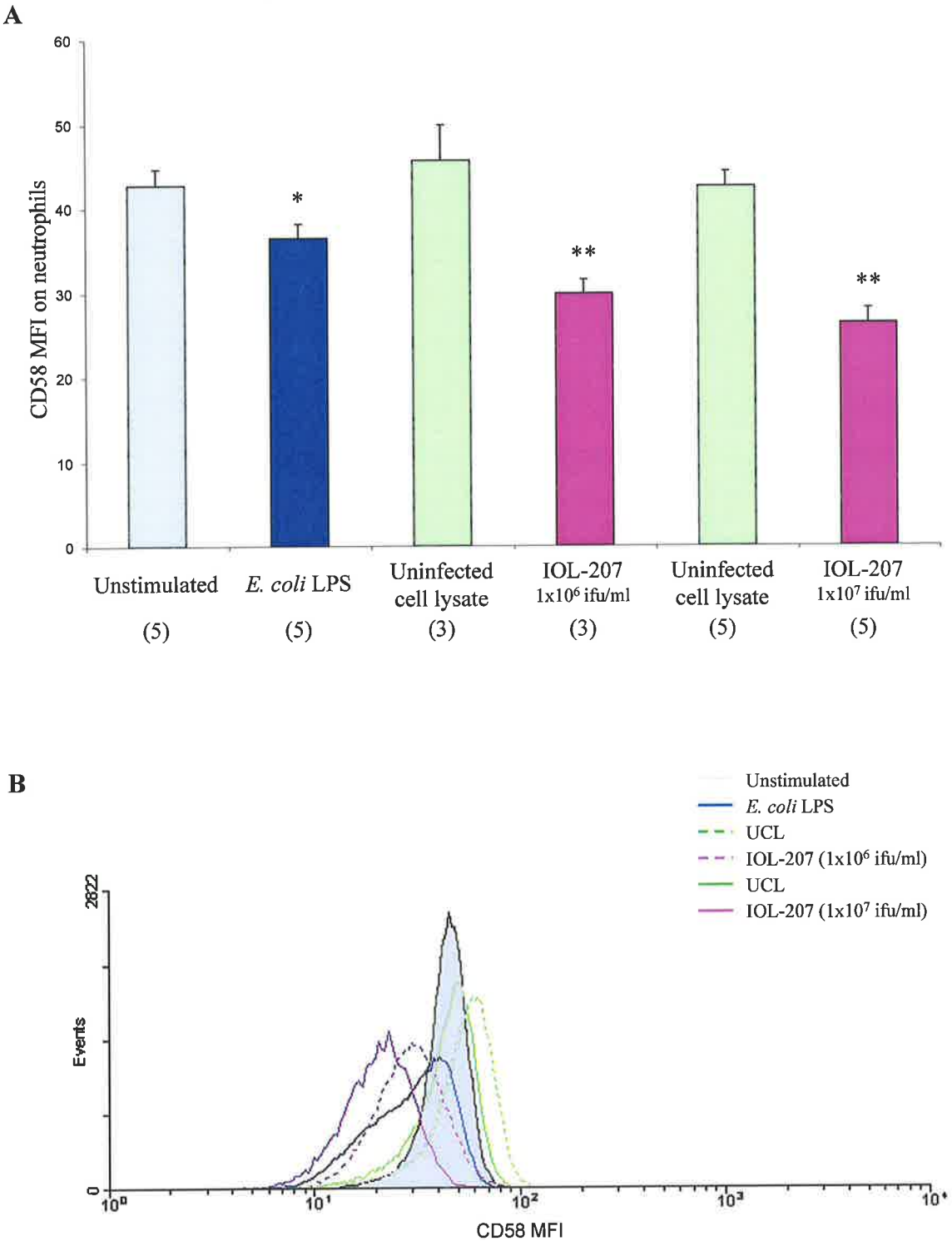


Figure 5.14: *C. pneumoniae* decreases CD58 expression on neutrophils.
Whole blood neutrophils were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD58 MFI was determined using an anti-human CD58 FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of neutrophils displaying decreased CD58 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

Percentage of neutrophils expressing CD11a						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	96.72	90.78	68.43	65.73	94.94	98.26
2	96.68	97.69	90.97	54.90	98.05	99.28
3	98.02	90.29	74.61	55.26	81.41	88.14
4	97.51	87.77	nd	nd	93.62	98.30
5	93.42	74.84	nd	nd	92.14	92.27
Mean	96.47	88.28*	78.00*	58.63**	92.03	95.25
SEM	0.80	3.74	6.72	3.55	2.83	2.17

Table 5.9: Effect of *C. pneumoniae* on the percentage of neutrophils expressing CD11a.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were selected based on forward and side scatter characteristics. CD11a expression was detected using an anti-human FITC labelled monoclonal CD11a antibody and detected using flow cytometry. Data represents the mean percentage of neutrophils expressing CD11a from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to comparable amount of UCL, mixed ANOVA.

When we analysed the MFI of neutrophils expressing CD11a, we found that stimulation with *E. coli* LPS decreased CD11a expression compared to unstimulated cells (**Figure 5.15**). Small but significant decreases in CD11a MFI were also observed after incubation of whole blood neutrophils with uninfected cell lysate. *C. pneumoniae* caused even further reduction of CD11a protein expression on the surface of neutrophils when compared to uninfected cell lysate.

Does C. pneumoniae modulate lymphocyte expression of adhesion molecules?
After determining that *C. pneumoniae* changes the expression of adhesion molecules on both monocytes and neutrophils we also aimed to examine whether the expression of these molecules and their ligands were also altered on lymphocytes. We first investigated the expression of ICAM-1 (which is also the ligand for CD11a on other cells) on lymphocytes and found that as little as 5% of resting lymphocytes were positive for ICAM-1 staining (**Table 5.10**). However, stimulation with PHA caused an increase in the percentage of lymphocytes expressing ICAM-1 and increased to 10%. This showed that although expression was low, it could be modulated by stimulus. Incubation of whole blood with

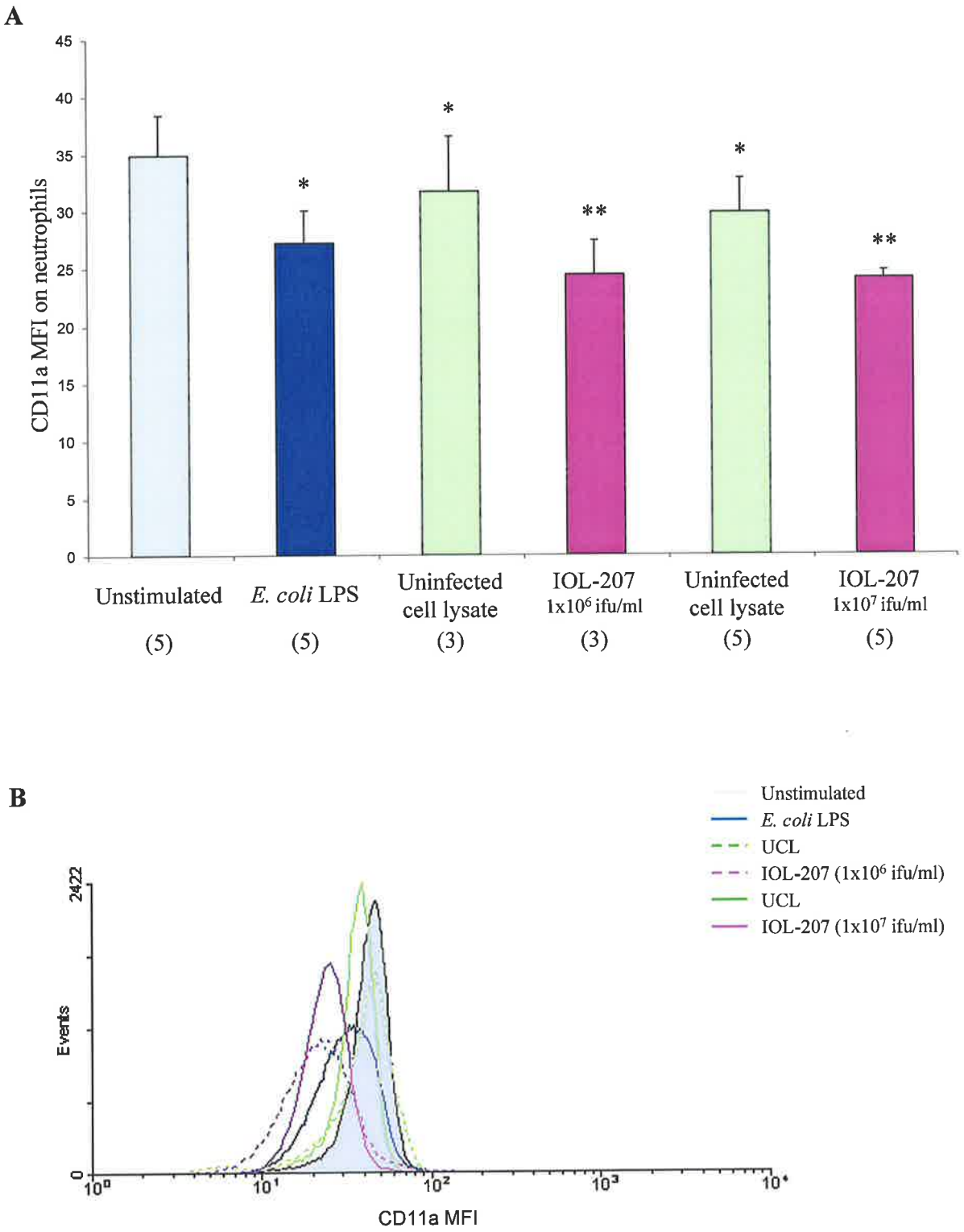


Figure 5.15: *C. pneumoniae* decreases CD11a expression on neutrophils.
Whole blood neutrophils were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD11a MFI was determined using an anti-human CD11a FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of neutrophils displaying decreased CD11a MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

uninfected cell lysate or *C. pneumoniae* failed to change the expression of ICAM-1 on lymphocytes compared to uninfected cells.

Percentage of lymphocytes expressing ICAM-1						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	4.57	9.75	4.52	6.01	3.90	4.00
2	3.26	9.23	3.46	5.98	4.48	5.25
3	7.86	11.71	7.57	5.23	4.53	4.23
Mean	5.23	10.23*	5.18	5.74	4.30	4.49
SEM	1.37	0.76	1.23	0.25	0.20	0.38

Table 5.10: *C. pneumoniae* does not effect the percentage of lymphocytes expressing ICAM-1. Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. ICAM-1 expression was detected using an anti-human PE labelled monoclonal CD54 antibody and detected using flow cytometry. Data represents the mean percentage of lymphocytes expressing ICAM-1 from 3 individual subjects, performed in triplicate. * $p<0.05$ compared to unstimulated cells, mixed ANOVA.

As the expression of ICAM-1 was relatively low and was unchanged by exposure to *C. pneumoniae*, we did not analyse the amount of ICAM-1 protein expression as defined by the MFI.

Similar to the observations with monocytes and neutrophils, CD11a was also constitutively expressed on lymphocytes with practically all lymphocytes staining positive fore this surface molecule (**Table 5.11**). The percentage of lymphocytes expressing CD11a was not altered under any of the conditions tested.

Percentage of lymphocytes expressing CD11a						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	99.88	99.95	99.89	99.94	99.76	99.78
2	99.71	99.86	99.70	99.90	99.24	99.75
3	98.11	98.32	96.34	97.56	97.75	97.34
Mean	99.23	99.38	98.64	99.13	98.92	98.96
SEM	0.56	0.53	1.15	0.79	0.60	0.81

Table 5.11: *C. pneumoniae* does not effect the percentage of lymphocytes expressing CD11a.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD11a expression was detected using an anti-human FITC labelled monoclonal CD11a antibody and detected using flow cytometry. Data represents the mean percentage of lymphocytes expressing CD11a from 3 individual subjects, performed in triplicate.

Analysis of the MFI of lymphocytes expressing CD11a showed that incubation of blood with PHA did not significantly change the MFI from unstimulated cells (**Table 5.12**). There was a significant decrease in lymphocyte CD11a MFI when blood was incubated with uninfected cell lysate but only reached statistical significance at the highest amount used. Stimulation of blood with *C. pneumoniae* caused an increase in CD11a MFI but only reached statistical significance at the lowest concentration which was equivalent to baseline levels of unstimulated cells.

MFI of lymphocytes expressing CD11a						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	122.96	137.62	119.07	134.33	106.33	104.64
2	91.58	99.00	82.79	92.65	70.32	79.51
3	65.97	64.96	57.97	61.12	57.57	53.80
Mean	93.50	100.53	86.61	96.03**	78.07*	79.32
SEM	16.48	20.99	17.74	21.20	14.60	14.68

Table 5.12: Effect of *C. pneumoniae* on the MFI of lymphocytes expressing CD11a.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD11a expression was detected using an anti-human FITC labelled monoclonal CD11a antibody and detected using flow cytometry. Data represents the mean MFI of lymphocytes expressing CD11a from 3 individual subjects, performed in triplicate. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of uninfected cell lysate (UCL), mixed ANOVA.

We have previously shown that monocytes and neutrophils express CD58 which binds to CD2 on lymphocytes. Therefore, we also assessed the expression of CD2 after exposure of lymphocytes to *C. pneumoniae*. We detected CD2 on the surface of practically all unstimulated lymphocytes (**Table 5.13**). Overnight incubation with any of the stimuli did not significantly alter the percentage of lymphocytes expressing CD2 and remained at 100%.

Percentage of lymphocytes expressing CD2						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	99.89	99.82	99.80	99.86	99.81	99.87
2	99.60	99.47	99.48	99.48	99.50	99.44
3	99.78	99.74	99.71	99.73	99.68	99.73
Mean	99.76	99.68	99.66	99.69	99.66	99.68
SEM	0.09	0.11	0.10	0.11	0.09	0.13

Table 5.13: *C. pneumoniae* does not effect the percentage of lymphocytes expressing CD2.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD2 expression was detected using an anti-human FITC labelled monoclonal CD2 antibody and detected using flow cytometry. Data represents the mean percentage of lymphocytes expressing CD2 from 3 individual subjects, performed in triplicate.

We then assessed whether the amount of CD2 protein expressed on the surface of lymphocytes was altered in response to exposure to *C. pneumoniae*. We initially observed that stimulation of whole blood with PHA caused a significant increase in the MFI of lymphocytes expressing CD2 (**Figure 5.16**). Incubation with uninfected cell lysate caused a small but significant decrease in CD2 MFI at the highest amount used in comparison to unstimulated cells. In addition, there was a small but significant increase in the MFI of lymphocytes expressing CD2 after stimulation with both concentrations of *C. pneumoniae* compared to blood incubated with uninfected cell lysate.

Cell surface expression of adhesion molecules mediates the interaction between leucocytes and surrounding structural tissues such as epithelium and endothelium. We have shown that a range of adhesion molecules are expressed on leucocytes derived from whole blood. We

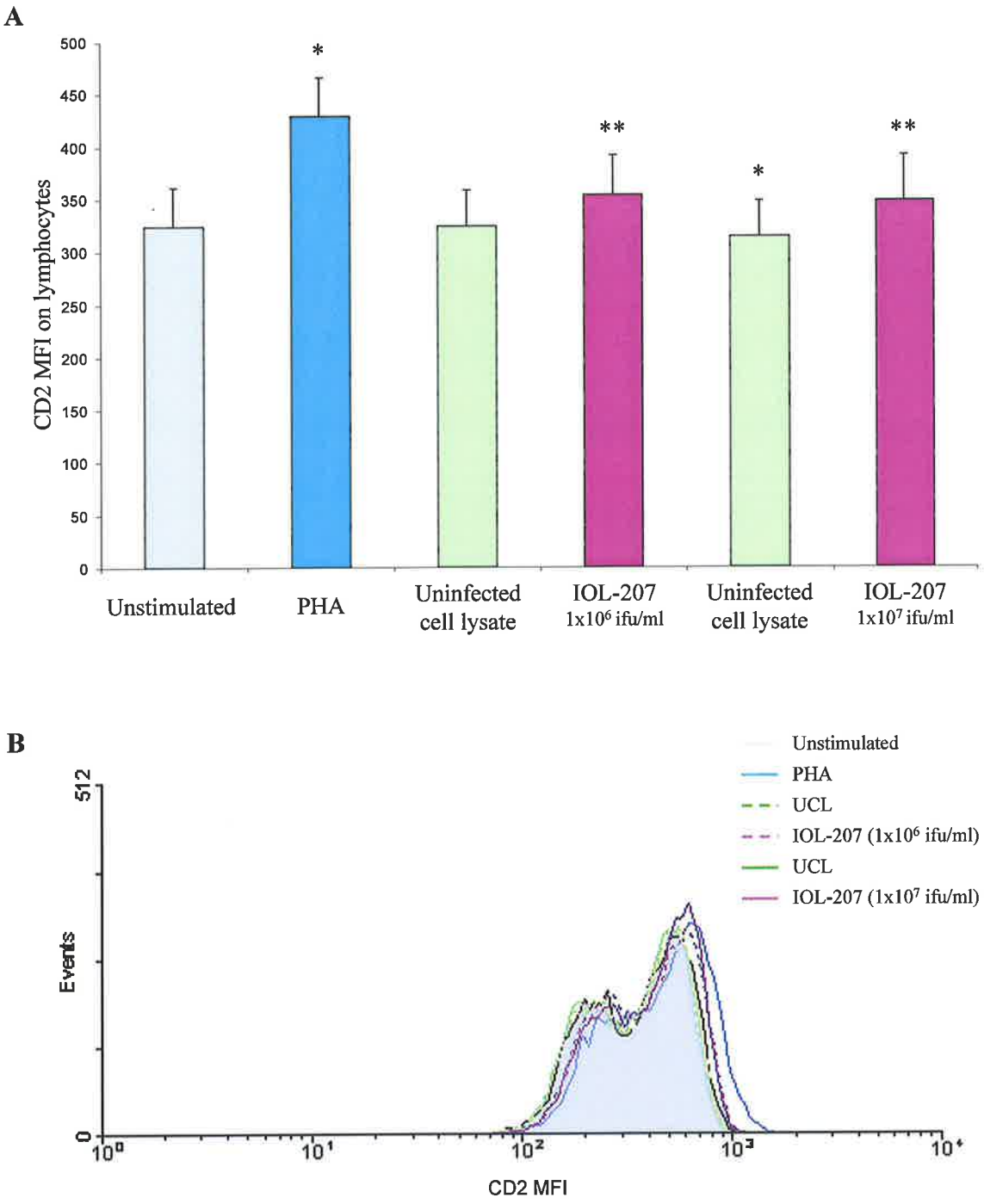


Figure 5.16: *C. pneumoniae* increases MFI of lymphocytes expressing CD2.
Whole blood was incubated overnight with PHA (2μg/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were gated based on $CD3^+$ staining and side scatter characteristics by flow cytometry and CD2 MFI was determined using an anti-human CD2 FITC labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of lymphocytes displaying decreased CD2 MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

assessed the expression of these molecules on monocytes, neutrophils and lymphocytes and found that *C. pneumoniae* exerts its affects on all cell types but predominantly monocytes and neutrophils. *C. pneumoniae* modulation of adhesion molecules examined within this chapter are summarised in **Table 5.14**.

Summary of adhesion molecule expression on leucocytes after *C. pneumoniae* exposure			
Surface molecule	**Monocyte**	**Neutrophil**	**Lymphocyte**
ICAM-1	↑	↑	↔
CD58	↔	↓	na
CD11a	↓	↓	↔
CD2	na	na	↑

Table 5.14: *C. pneumoniae* alters the expression of adhesion molecules on leucocytes.
na, not assessed; ↑, increased; ↔, unchanged; ↓, decreased.

Does C. pneumoniae alter the antigen presentation of monocytes through modulation of MHC molecule expression?

The second step in mounting an efficient T cell immune response is antigen presentation, which is mediated via expression of MHC molecules by antigen presenting cells. We therefore asked the question whether *C. pneumoniae* compromises the immune response by altering the level of MHC molecule expression by monocytes. We found that MHC Class I molecules were expressed on the entire population of unstimulated monocytes and this was not altered after overnight incubation with any of the cell treatments tested (**Table 5.15**).

Percentage of monocytes expressing MHC Class I molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	100.00	100.00	99.99	99.99	100.00	99.92
2	99.99	100.00	100.00	99.72	100.00	99.77
3	100.00	100.00	100.00	100.00	100.00	100.00
4	99.98	100.00	nd	nd	100.00	100.00
5	99.99	99.98	nd	nd	99.99	99.33
Mean	99.99	100.00	100.00	99.90	100.00	99.80
SEM	0.00	0.00	0.00	0.09	0.00	0.13

Table 5.15: *C. pneumoniae* does not effect the percentage of monocytes expressing MHC Class I molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. MHC Class I expression was detected using an anti-human FITC labelled monoclonal HLA-ABC antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing MHC Class I molecules from 5 individual subjects, performed in triplicate. nd: not done.

We then assessed the amount of MHC Class I protein expression on the cell surface by measuring the MFI of monocytes expressing these molecules. In comparison to unstimulated cells, *E. coli* LPS significantly increased the MFI of monocytes expressing MHC Class I molecules (**Table 5.16**). The highest amount of uninfected cell lysate also resulted in a significant increase in MHC Class I MFI compared to unstimulated cells. After stimulation of whole blood with the lowest concentration of *C. pneumoniae*, monocyte MHC Class I MFI was not significantly different compared to blood incubated with the lowest amount of uninfected cell lysate. In contrast, the highest concentration of *C. pneumoniae* caused a significant decrease in MHC Class I MFI in comparison to the appropriate amount of uninfected cell lysate, the level of which, was similar to unstimulated blood.

MFI of monocytes expressing MHC Class I molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	558.39	845.07	469.86	527.79	587.36	404.80
2	492.31	666.08	359.11	335.27	918.11	602.75
3	689.13	891.76	460.06	517.12	744.14	503.71
4	293.39	1041.23	nd	nd	648.16	423.60
5	939.18	1209.54	nd	nd	1282.74	923.39
Mean	594.48	930.74*	429.68	460.06	836.10*	571.65**
SEM	107.28	91.88	35.40	62.47	124.87	94.62

Table 5.16: Effect of *C. pneumoniae* on the MFI of monocytes expressing MHC Class I molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. MHC Class I expression was detected using an anti-human FITC labelled monoclonal HLA-ABC antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing MHC Class I molecules from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to UCL, mixed ANOVA.

Similar results were obtained when examining the expression of MHC Class II molecules on monocytes, in that all monocytes constitutively expressed MHC Class II molecules and remained at this level under all conditions tested (**Table 5.17**).

Percentage of monocytes expressing MHC Class II molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	99.82	99.83	99.23	99.93	99.48	99.14
2	97.74	99.67	95.44	98.71	99.95	99.74
3	99.80	99.74	93.72	99.91	99.57	99.97
4	99.94	99.93	nd	nd	99.63	99.94
5	99.10	99.44	nd	nd	99.87	100.00
Mean	99.28	99.72	96.13	99.52	99.70	99.76
SEM	0.41	0.08	1.63	0.40	0.09	0.16

Table 5.17: *C. pneumoniae* does not effect the percentage of monocytes expressing MHC Class II molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. MHC Class II expression was detected using an anti-human FITC labelled monoclonal HLA-DR antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing MHC Class II molecules from 5 individual subjects, performed in triplicate. nd: not done.

We then assessed the amount of MHC Class II protein expression on the cell surface by measuring the MFI of monocytes expressing these molecules. Similar to monocyte expression of MHC Class I expression, treatment of whole blood with *E. coli* LPS significantly increased MHC Class II MFI (**Table 5.18**). Incubation of blood with a the lowest amount of uninfected cell lysate caused a significant decrease in monocyte MHC Class II MFI in comparison to unstimulated blood but resulted in a significant increase at the higher amount. *C. pneumoniae* stimulation of whole blood with a low concentration resulted in a small but significant increase in MFI of monocytes expression MHC Class II molecules but had no effect at the highest concentration used.

MFI of monocytes expressing MHC Class II molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	415.42	521.31	256.39	366.71	319.38	323.01
2	304.85	322.65	236.39	294.52	303.41	399.37
3	293.84	406.93	229.46	362.28	347.85	426.95
4	482.61	733.78	nd	nd	735.03	385.98
5	450.97	644.14	nd	nd	598.04	523.29
Mean	389.54	525.76*	240.75*	341.17**	460.74*	411.72
SEM	38.36	75.06	8.07	23.36	87.05	32.68

Table 5.18: Effect of *C. pneumoniae* on the MFI of monocytes expressing MHC Class II molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. MHC Class II expression was detected using an anti-human FITC labelled monoclonal HLA-DR antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing MHC Class II molecules from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to UCL, mixed ANOVA.

Does C. pneumoniae alter the antigen presentation of neutrophils through modulation of MHC molecule expression?

Although neutrophils are not typically described as antigen presenting cells they do express MHC molecules and we therefore investigated whether exposure to *C. pneumoniae* changed the level of expression of these molecules. We were able to consistently detect MHC Class I molecules on neutrophils from unstimulated whole blood cultures (**Table 5.19**). Stimulation with *E. coli* LPS had no effect on the percentage of neutrophils expressing MHC Class I

molecules and was maintained at 100%. Likewise, when whole blood was incubated overnight with uninfected cell lysate or *C. pneumoniae*, the percentage of neutrophils staining positively for MHC Class I molecules was maintained on the entire population of selected neutrophils.

Percentage of neutrophils expressing MHC Class I molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	97.97	92.50	99.39	99.38	96.59	97.56
2	99.63	99.02	99.64	98.85	99.90	98.84
3	98.94	99.09	97.91	75.03	99.51	96.28
4	98.19	98.68	nd	nd	98.89	99.36
5	90.41	98.98	nd	nd	99.83	96.65
Mean	97.03	97.65	98.98	91.09	98.94	97.74
SEM	1.68	1.29	0.54	8.03	0.61	0.60

Table 5.19: *C. pneumoniae* does not effect the percentage of neutrophils expressing MHC Class I molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were selected based on forward and side scatter characteristics. MHC Class I expression was detected using an anti-human FITC labelled monoclonal HLA-ABC antibody and detected using flow cytometry. Data represents the mean percentage of neutrophils expressing MHC Class I molecules from 5 individual subjects, performed in triplicate. nd: not done.

We then analysed the MFI of neutrophils expressing MHC Class I molecules and found that low amounts of uninfected cell lysate caused a significant decrease in the MFI. Although incubation with the lowest concentration of *C. pneumoniae* also caused a decrease in MHC Class I MFI, it did not significantly differ from those cells that were exposed to uninfected cell lysate (**Table 5.20**). Higher amounts of uninfected cell lysate also significantly decreased the MFI of neutrophils expressing MHC Class I molecules, compared to unstimulated cells. However, in contrast to low concentrations of *C. pneumoniae*, the highest concentration of *C. pneumoniae* ($1x10^7$ ifu/ml) caused a further reduction of MHC Class I MFI on neutrophils, which was equivalent to neutrophils incubated with $1x10^6$ ifu/ml and the lower concentration of uninfected cell lysate. It is therefore difficult to assess whether the decrease in MHC Class I MFI with $1x10^7$ ifu/ml was a true result caused by the bacteria.

MFI of neutrophils expressing MHC Class I molecules						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	149.58	150.90	82.23	90.92	92.10	69.74
2	102.33	96.66	78.12	54.96	89.47	76.88
3	100.10	91.69	73.21	65.30	94.97	66.57
4	46.83	76.53	nd	nd	63.19	50.53
5	220.78	198.08	nd	nd	191.60	114.67
Mean	123.92	122.77	77.86*	70.39	106.26*	75.68**
SEM	29.17	22.64	2.61	10.69	22.08	10.66

Table 5.20: Effect of *C. pneumoniae* on the MFI of neutrophils expressing MHC Class I molecules. Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics and MHC Class I expression was detected using an anti-human FITC labelled monoclonal HLA-ABC antibody and detected using flow cytometry. Data represents the mean MFI of neutrophils expressing MHC Class II molecules from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to UCL, mixed ANOVA.

We also analysed neutrophils for the expression of MHC Class II molecules. We did not detect MHC Class II expression on unstimulated cells and it was not induced after overnight stimulation of whole blood with LPS. In addition, MHC Class II expression was not induced by *C. pneumoniae*.

The expression of MHC molecules plays an important role in antigen presentation to lymphocytes. We assessed the expression of these molecules on professional antigen presenting cells and observed that *C. pneumoniae* has little effect on the expression of these molecules on monocytes. In comparison, neutrophils only expressed MHC Class I molecules, which were down regulated by uninfected cell lysate and after stimulation of whole blood with *C. pneumoniae*. A summary of these results is displayed in **Table 5.21**.

Summary of MHC molecule expression on leucocytes after *C. pneumoniae* exposure			
Surface molecule	**Monocyte**	**Neutrophil**	**Lymphocyte**
MHC Class I	↔	↓	na
MHC Class II	↔	nd	na

Table 5.21: Summary of MHC molecule expression on leucocytes after exposure to *C. pneumoniae*. ↔, unchanged; ↓, decreased; nd, not detected; na, not assessed.

Does C. pneumoniae alter monocyte expression of surface molecules involved in costimulation?

After adhesion and antigen presentation, activation of T cells still requires an additional costimulatory signal which is predominantly provided through the expression of B7 molecules CD80 and CD86. Therefore, we assessed the expression of both CD80 and CD86 expression on whole blood monocytes after exposure to *C. pneumoniae*.

Unstimulated monocytes did not express CD80 on their cell surface but it was significantly induced on 74% of cells after *E. coli* LPS stimulation (**Figure 5.17**). In comparison to unstimulated cells, whole blood incubated with uninfected cell lysate also resulted in an increase in the percentage of monocytes expressing CD80 (20 – 56%). However, after exposure to *C. pneumoniae*, the percentage of monocytes expressing CD80 was significantly reduced to 10% and 22% of monocytes respectively, in comparison to uninfected cell lysate.

The MFI of monocytes expressing CD80 was also determined using flow cytometry and showed that *E. coli* LPS significantly increased CD80 MFI (**Table 5.22**). A similar effect was observed when cells were incubated with uninfected cell lysate, which also showed increased CD80 MFI on monocytes compared to unstimulated cells. However, exposure of monocytes to *C. pneumoniae* did not significantly alter CD80 MFI on monocytes in comparison to the already upregulated expression observed with uninfected cell lysate.

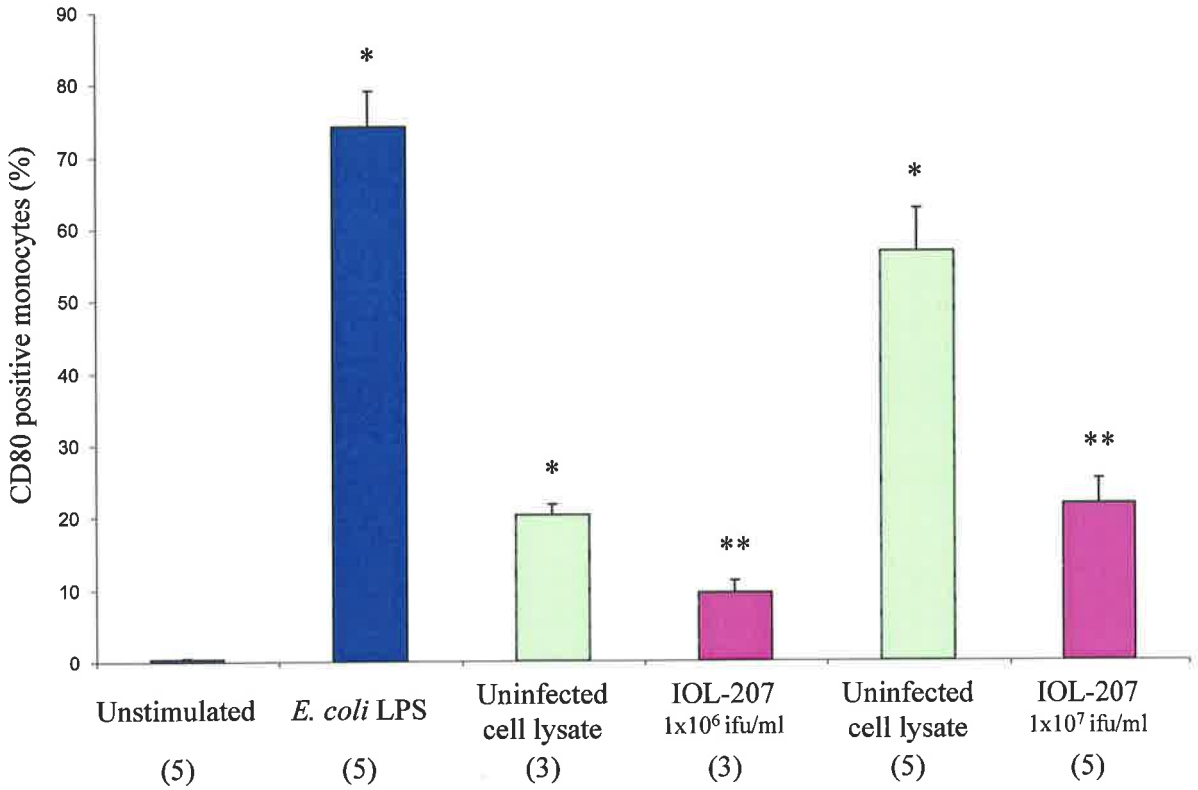


Figure 5.17: *C. pneumoniae* induces low but significant monocyte CD80 expression.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^{+}$ antibody staining by flow cytometry and CD80 positive cells were detected using an anti-human CD80 PE labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

MFI of monocytes expressing CD80						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	60.31	153.69	182.61	166.46	129.13	96.93
2	80.20	118.43	118.58	103.75	122.29	125.40
3	64.76	144.66	135.48	165.23	142.80	97.43
4	86.20	224.05	nd	nd	135.47	194.09
5	61.81	151.15	nd	nd	94.31	69.03
Mean	70.66	158.40*	145.56*	145.15	124.80*	116.58
SEM	5.26	17.57	19.16	20.70	8.34	21.33

Table 5.22: *C. pneumoniae* does not effect the MFI of monocytes expressing CD80.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. CD80 MFI was determined using an anti-human PE labelled monoclonal antibody to CD80 and detected using flow cytometry. Data represents the mean MFI of monocytes expressing CD80 molecules from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA.

Costimulation signals are also provided by monocytes through the expression of CD86. CD86 expression was constitutively expressed on approximately 70% of unstimulated monocytes (**Table 5.23**). After incubation of whole blood with *E. coli* LPS, the percentage of monocytes expressing CD86 was significantly reduced compared to unstimulated cells. Incubation of whole blood with the highest amount of uninfected cell lysate caused a small but significant decrease in the percentage of monocytes expressing CD86. The effect of *C. pneumoniae* on the percentage of monocytes expressing CD86 is variable. At the lowest concentration ($1x10^6$ ifu/ml) CD86 expression was decreased, but at $1x10^7$ ifu/ml the number of monocytes expressing CD86 was increased, when compared to uninfected cell lysate.

Percentage of monocytes expressing CD86						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	81.44	62.64	67.05	50.30	61.05	82.59
2	67.68	57.46	75.16	54.52	58.49	66.42
3	64.58	36.61	73.26	47.54	36.98	56.55
4	91.24	72.21	nd	nd	91.08	80.46
5	39.13	29.38	nd	nd	22.06	23.00
Mean	68.81	51.66*	71.82	50.79**	53.93*	61.80**
SEM	8.84	8.06	2.45	2.03	11.74	10.80

Table 5.23: Effect of *C. pneumoniae* on the percentage of monocytes expressing CD86.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. CD86 MFI was detected using an anti-human PE labelled monoclonal CD86 antibody and detected using flow cytometry. Data represents the mean percentage of monocytes expressing CD86 from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$, compared to unstimulated, mixed ANOVA. ** $p<0.05$, compared to UCL, mixed ANOVA.

The MFI of monocytes expressing CD86 were also examined after stimulation of whole blood with *C. pneumoniae*. The MFI of unstimulated monocytes expressing CD86 was relatively high and was significantly reduced after exposure to *E. coli* LPS (**Figure 5.18**). Uninfected cell lysate had a similar effect and MFI was significantly reduced compared to unstimulated cells. *C. pneumoniae* stimulation of monocytes was significantly decreased at the lowest concentration ($1x10^6$ ifu/ml) when compared to the appropriate amount of uninfected cell lysate. Monocyte CD86 MFI was also reduced at the higher concentration of *C. pneumoniae* compared to blood incubated with uninfected cell lysate but this did not reach statistical significance (possibly due to variability).

The expression of a third costimulatory molecule, CD40, was also examined on monocytes in response to *C. pneumoniae* exposure. The percentage of unstimulated monocytes expressing CD40 was minimal but significantly induced on approximately 40% of cells stimulated with *E. coli* LPS (**Figure 5.19**). Incubation of cells with the lowest amount of uninfected cell lysate and lowest concentration of *C. pneumoniae* ($1x10^6$ ifu/ml) failed to induce monocyte

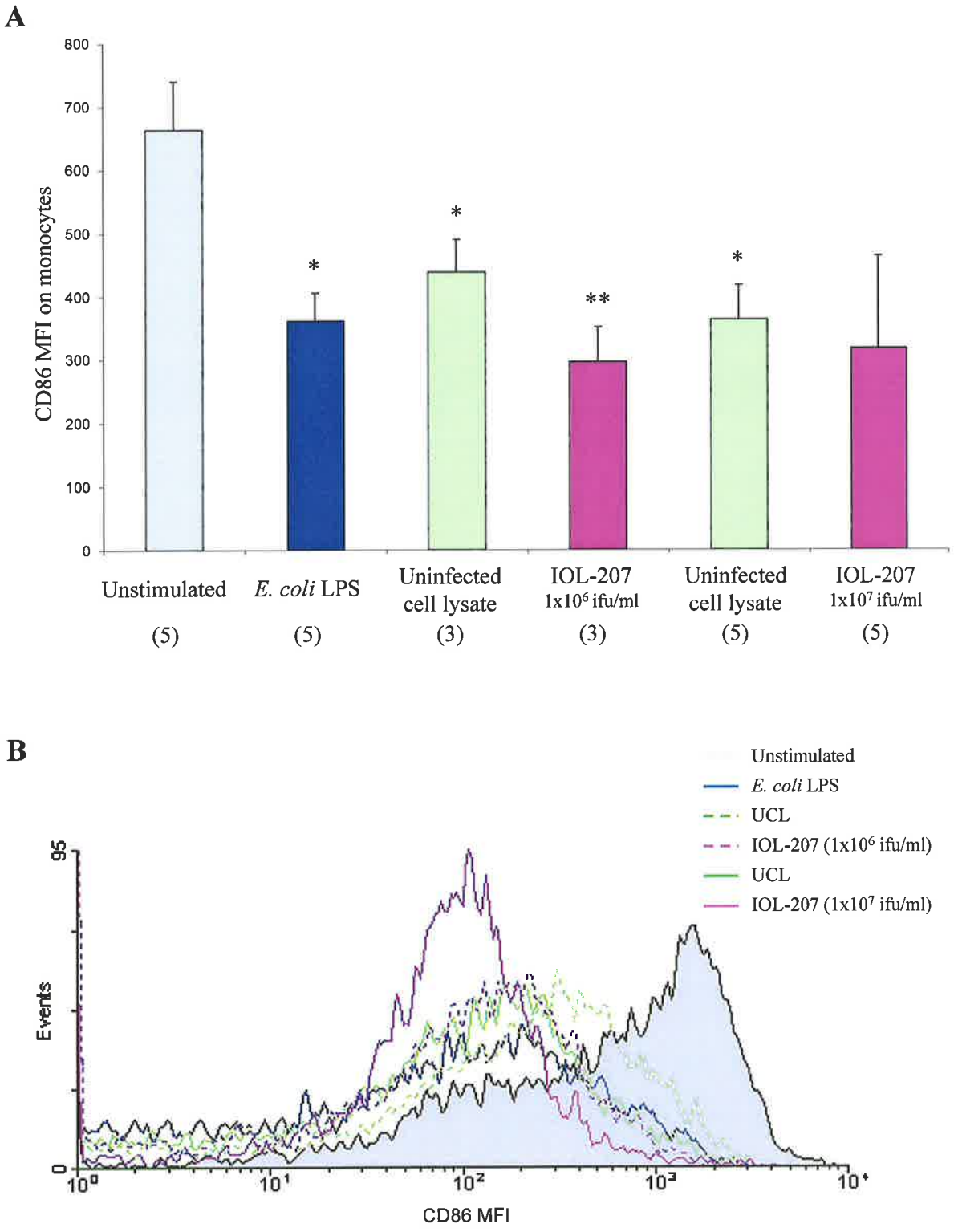


Figure 5.18: Effect of *C. pneumoniae* stimulation on MFI of monocytes expressing CD86.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ staining and side scatter characteristics by flow cytometry and CD86 MFI was determined using an anti-human CD86 PE labelled monoclonal antibody. **A)** Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. **B)** Histogram is representative of monocytes displaying CD86 MFI after *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

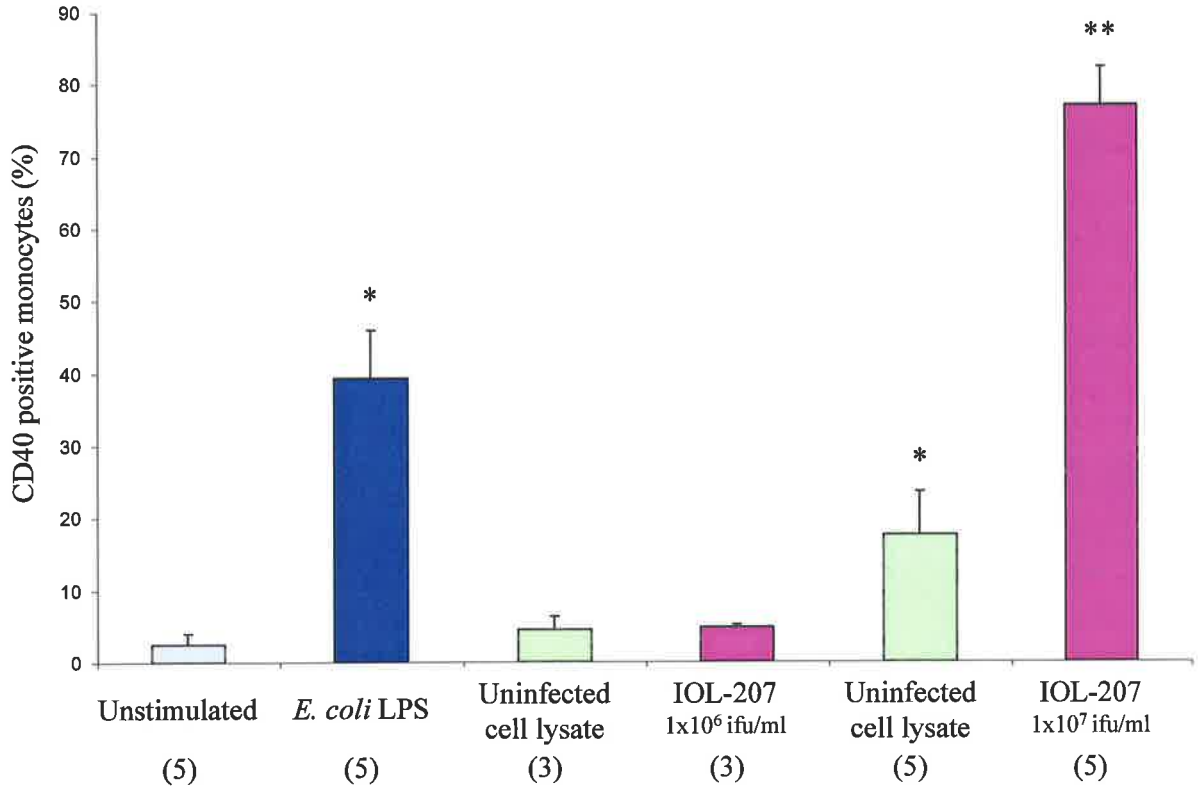


Figure 5.19: *C. pneumoniae* increases the percentage of monocytes expressing CD40.
Whole blood monocytes were incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ antibody staining by flow cytometry and CD40 positive cells were detected using an anti-human CD40 PE labelled monoclonal antibody. Bars represent the mean ± SEM of (3) or (5) separate experiments performed in triplicate. * $p<0.05$, compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

CD40 expression. In contrast, the higher amount of uninfected cell lysate induced CD40 expression on approximately 20% of monocytes which was significantly increased further after exposure to *C. pneumoniae* at the highest concentration ($1x10^7$ ifu/ml).

The MFI of monocytes expressing CD40 was not significantly different between unstimulated cells and those stimulated with *E. coli* LPS (**Table 5.24**). Incubation of whole blood with the lower amount of uninfected cell lysate caused a significant increase in CD40 MFI, whereas, the higher amount cause a significant decrease, in comparison to unstimulated whole blood. Contrasting results were also observed when whole blood was stimulated with *C. pneumoniae* where $1x10^6$ ifu/ml did not alter CD40 MFI compared to the MFI already increased by a comparable amount of uninfected cell lysate. However, whole blood stimulation with $1x10^7$ ifu/ml of *C. pneumoniae* caused an increase in the MFI of monocytes expressing CD40 compared to a comparable amount of uninfected cell lysate, returning to baseline expression of unstimulated blood.

MFI of monocytes expressing CD40						
Subject	**Unstimulated**	***E. coli* LPS (100ng/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	114.68	200.71	146.09	160.45	73.25	78.93
2	190.24	108.37	117.53	105.73	39.25	50.73
3	93.91	252.33	179.50	157.82	64.18	88.20
4	76.79	71.64	nd	nd	64.62	70.09
5	34.24	36.48	nd	nd	42.85	111.55
Mean	101.97	133.90	147.71*	141.33	56.83*	79.90**
SEM	25.73	40.32	17.91	17.82	6.67	10.05

Table 5.24: *C. pneumoniae* does not effect the MFI of monocytes expressing CD40.
Whole blood was incubated overnight with *E. coli* LPS, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Monocytes were selected based on $CD14^+$ staining and side scatter characteristics. CD40 MFI was determined using an anti-human PE labelled monoclonal antibody to CD40 and detected using flow cytometry. Data represents the mean MFI of monocytes expressing CD40 molecules from 5 individual subjects, performed in triplicate. nd: not done. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to UCL, mixed ANOVA. Raw data was logged before applying the mixed ANOVA model for analysis.

Does C. pneumoniae alter neutrophil expression of surface molecules involved in costimulation?

The classical costimulatory molecules from the B7 family, CD80 and CD86 were not detected on neutrophils. In addition, after exposure to any of the agents used in these experiments, their expression remained undetected. Similarly, the expression of CD40 on neutrophils was not detected, nor was it induced under any treatments tested.

Does C. pneumoniae alter lymphocyte expression of surface molecules involved in costimulation?

The B7 family of costimulatory molecules bind to CD28 on lymphocytes. We therefore asked whether *C. pneumoniae* modulated the expression of CD28 on lymphocytes. As can be seen in **Table 5.25**, CD28 was constitutively expressed on approximately 80% of lymphocytes. Overnight incubation with any of the treatments, including PHA, uninfected cell lysate or *C. pneumoniae*, had no effect on the percentage of lymphocytes staining positively for CD28.

Percentage of lymphocytes expressing CD28						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* $1x10^6$**	**UCL**	***C. pneumoniae* $1x10^7$**
1	64.33	65.19	64.05	61.48	63.85	60.92
2	94.06	94.36	94.28	93.47	94.43	93.46
3	77.41	77.39	77.86	77.96	77.54	77.32
Mean	78.60	78.98	78.73	77.64	78.61	77.24
SEM	8.60	8.46	8.74	9.24	8.84	9.39

Table 5.25: *C. pneumoniae* does not effect the percentage of lymphocytes expressing CD28.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD28 expression was detected using an anti-human PE labelled monoclonal CD28 antibody and detected using flow cytometry. Data represents the mean percentage of lymphocytes expressing CD28 from 3 individual subjects, performed in triplicate.

We also assessed whether the amount of CD28 protein being expressed by lymphocytes was altered by stimulation. After overnight stimulation of whole blood with PHA, the basal level of CD28 expression, as determined by analysis of the MFI of lymphocytes expressing CD28, increased significantly when compared to unstimulated cells (**Table 5.26**). Uninfected cell

lysate caused a small but significant decrease in CD28 MFI at the highest amount used. *C. pneumoniae* also caused a slight decrease in CD28 MFI of lymphocytes compared to uninfected cell lysate but this did not reach statistical significance.

MFI of lymphocytes expressing CD28						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	356.70	377.38	340.54	327.87	311.66	327.42
2	440.51	456.20	419.30	425.18	397.80	337.46
3	326.09	370.17	299.83	283.69	278.75	259.57
Mean	374.43	401.25*	353.22	345.58	329.40*	308.15
SEM	34.20	27.55	35.07	41.79	35.49	24.46

Table 5.26: Effect of *C. pneumoniae* on the MFI of lymphocytes expressing CD28.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD28 expression was detected using an anti-human PE labelled monoclonal CD28 antibody and detected using flow cytometry. Data represents the mean MFI of lymphocytes expressing CD28 from 3 individual subjects, performed in triplicate. * $p<0.05$, compared to unstimulated, mixed ANOVA.

We also investigated the expression of CD40L on lymphocytes, as we had previously shown that stimulation of monocytes with *C. pneumoniae* increased CD40 expression. We found that the expression of CD40L on lymphocytes from unstimulated whole blood was barely detectable (**Table 5.27**). Stimulation with PHA significantly increased this expression to approximately 5%. Incubation with uninfected cell lysate or *C. pneumoniae* failed to change the percentage of lymphocytes expressing CD40L, which remained at less than 2%.

Percentage of lymphocytes expressing CD40L						
Subject	**Unstimulated**	**PHA (2μg/ml)**	**UCL**	***C. pneumoniae* 1x10^6**	**UCL**	***C. pneumoniae* 1x10^7**
1	1.03	2.04	1.12	0.88	0.53	0.85
2	1.94	3.74	2.30	0.88	0.81	1.04
3	1.23	4.32	1.52	0.62	0.73	0.56
Mean	1.40	3.37	1.65	0.79	0.69	0.82
SEM	0.28	0.69	0.35	0.09	0.08	0.14

Table 5.27: *C. pneumoniae* does not change the percentage of lymphocytes expressing CD40L.
Whole blood was incubated overnight with PHA, uninfected cell lysate (UCL) or *C. pneumoniae* (IOL-207). Lymphocytes were selected based on $CD3^+$ staining and side scatter characteristics. CD40L expression was detected using an anti-human PE labelled monoclonal CD40L antibody and detected using flow cytometry. Data represents the mean percentage of lymphocytes expressing CD40L from 3 individual subjects, performed in triplicate.

As the percentage of lymphocytes expressing CD40L was very low and unchanged after exposure to *C. pneumoniae*, we did not analyse the amount of CD40L protein expression as defined by the MFI.

The modulation of expression of molecules involved in costimulatory processes by *C. pneumoniae* are summarised in **Table 5.28**.

***C. pneumoniae* modulation of leucocyte expression of surface molecules involved in costimulation**			
Surface molecule	**Monocyte**	**Neutrophil**	**Lymphocyte**
CD80	↑	nd	na
CD86	↔	nd	na
CD40	↑	nd	na
CD28	na	na	↔
CD40L	na	na	↔

Table 5.28: *C. pneumoniae* alters the expression of molecules involved in costimulation on leucocytes.
nd, not detected; na, not assessed; ↑, increased; ↔, unchanged.

Discussion

In the current chapter we have extended our findings investigating the host cell-pathogen relationship between peripheral blood leucocytes and *C. pneumoniae*. Within our first set of aims, we examined whether *C. pneumoniae* activated leucocytes, derived from whole blood, as determined by the expression of activation markers. The response of inflammatory cells to *C. pneumoniae* in terms of activation markers, specifically CD69 and CD25 expression, has not previously been described.

CD69 is classified as an early activation marker and is rapidly expressed on T lymphocytes after stimulation via the T cell receptor complex (CD3), CD2 or protein kinase C activation (328,329,430). The ligand for CD69 is currently unknown, however crosslinking with antibodies on lymphocytes, monocytes and neutrophils all result in an influx of Ca^{2+} and is a hallmark of cellular activation (328,431-433). The cytotoxic activity of monocytes is also increased via expression and crosslinking of CD69 and leads to the release of inflammatory mediators including leukotrienes and prostaglandins (433). Nitric oxide release by monocytes is also increased after CD69 crosslinking (434). These mediators are known to regulate immune and inflammatory processes including regulation of cytokines and cytokine receptor expression (435,436). Further, prostaglandin release by monocytes, inhibits IL-2 and IFN-γ in lymphocytes and promotes Th2 cytokine patterns by increasing IL-5 (437,438).

Ligation of CD69 on neutrophils causes an influx of Ca^{2+} and is thought to be involved in neutrophil degranulation causing release of granule contents including lysozyme (432). There is also some evidence that crosslinking of CD69 on neutrophils may be involved in cytokine production from neutrophils, synergising with GM-CSF and LPS (439). CD69 ligation also increases NK cell cytotoxicity (440). These processes are key inflammatory mechanisms important in host defence.

Lymphocytes respond to CD69 ligation by increasing TNF-α, IFN-γ, IL-2, CD25 expression leading to T cell proliferation but this is dependent on simultaneous protein kinase C activation (328,441). Crosslinking of CD69 on T cells also causes an increase in AP-1 and NF-AT transcription factor binding activity which are both involved in IL-2 production by T lymphocytes (442,443). IL-2 is a key cytokine involved in the maturation and proliferation of T cells, NK cells and monocytes, vital to the defence against potential pathogens (159). It also increases IL-1 expression, phagocytosis and cytotoxic activity of monocytes (159).

We found that unstimulated lymphocytes did not express CD69 but it was induced on cells stimulated with PHA, consistent with previous studies (441,444). In contrast to unstimulated lymphocytes, we detected up to 18% of monocytes from unstimulated cultures constitutively expressed CD69 on their surface which is also consistent with previous publications (433,444). We detected a low but easily detectable population of neutrophils that expressed CD69 in unstimulated cultures. Although unstimulated neutrophils isolated immediately from whole blood do not express CD69 on their surface it can be induced after overnight incubation in media alone (445). Incubation of whole blood cultures with *E. coli* LPS significantly increased CD69 expression on the surface of both monocytes and neutrophils compared with unstimulated cells. Other reports have also shown an upregulation of CD69 surface expression upon activation of monocytes and neutrophils (432,433).

After confirming the expression of CD69 on stimulated lymphocytes, monocytes and neutrophils, we next assessed the cellular response to *C. pneumoniae* stimulation. *C. pneumoniae* increased the expression of CD69 on monocytes, neutrophils and lymphocytes. The percentage of lymphocytes and monocytes expressing CD69 was increased after stimulation with *C. pneumoniae*, compared to uninfected cell lysate and this was a consistent pattern also reflected in the MFI data. The number of neutrophils expressing CD69 was increased after exposure to *C. pneumoniae* but the MFI of these cells did not show any

changes significantly different to cells incubated with media alone or uninfected cell lysate. Resting neutrophils contain an intracellular store of CD69 protein, that is mobilised to the cell surface upon activation (446). Overnight incubation of whole blood in media alone resulted in approximately 10% of neutrophils expressing CD69. Stimulation of blood with *C. pneumoniae* caused the number of neutrophils expressing CD69 to be over and above that of cells incubated with uninfected cell lysate or unstimulated cells incubated with media alone.

Our data showing an increase in CD69 expression on peripheral blood leucocytes after *C. pneumoniae* stimulation, has not been previously reported and suggests that *C. pneumoniae* may play a role in the activation of these cells. *C. pneumoniae* induced activation of host defence pathways by CD69 expression and ligation may be important in the initial host cell response to eradicate infection.

The modulation of CD69 expression by *C. pneumoniae* on leucocytes may be mediated via a variety of mechanisms. A number of cytokines are shown to modulate CD69 expression. Neutrophils stimulated with GM-CSF, IFN-γ or IFN-α induces CD69 surface expression (439,445). Recent reports demonstrate that monocytes respond to GM-CSF, IFN-γ, IFN-β and IL-1β by increasing CD69 expressing to varying intensities (447). Although monocytes and T cells are a source of these cytokines, in our previous chapter we did not detect IFN-γ production and did not assess GM-CSF expression after *C. pneumoniae*. Although it is unlikely that IFN-γ was present in the whole blood cultures, it does not completely exclude the contribution of these cytokines to CD69 induction by *C. pneumoniae*. In the studies in the previous chapter analysing cytokine expression by leucocytes in response to *C. pneumoniae*, a golgi blocking agent (Brefeldin A) was added to whole blood to allow detection of intracellular cytokine expression. In order to assess surface molecule expression the addition of a golgi block would prevent the mobilisation of proteins from the golgi apparatus to the

cell surface and therefore was not added in the current studies, thus also allowing secretion of cytokines. It is possible that CD69 expression was influenced by the presence of IL-1β in whole blood cultures. Other studies have shown that monocytes increase their production of IL-1β after *C. pneumoniae* infection (250). The expression of these cytokines, GM-CSF, IFN-γ and IL-1 are also altered in patients with chronic airways diseases such as asthma and COPD (222,301). The modulation of cytokine networks in chronic inflammatory states may be a contributing factor toward the upregulation of CD69 detected in asthmatics and COPD patients (314,448,449). This may suggest that *C. pneumoniae*, known to be associated with states of chronic airways disease, may in part contribute to the production of cytokines by leucocytes which then impacts on the expression of CD69.

It is possible that CD69 upregulation by *C. pneumoniae* may be due to an alternate mechanism, involving transcription factor activation. The CD69 promoter contains potential binding sites for various transcription factors and includes consensus sequences for NFκB, Oct-1/Oct-2 and transcription factors belonging to the ets and GATA families (450,451). These consensus sequences are located within a phorbol ester responsive region of the CD69 promoter. It is well documented that *C. pneumoniae* infection increases the activity of NFκB in various cell types including epithelial and endothelial cells (127,141). There is little information however, regarding NFκB activity in cells of the haematopoietic lineage with respect to *C. pneumoniae* infection. One study using a monocytic cell line, Mono Mac 6, showed an increase in NFκB binding activity after *C. pneumoniae* infection (422). It is therefore possible that the induction of CD69 in monocytes and possibly lymphocytes and neutrophils is a result of increased transcription factor activity.

In addition to CD69, we also investigated the expression of another cell surface marker indicative of cellular activation, CD25, by leucocytes in response to *C. pneumoniae*. The

overall response of monocytes and lymphocytes to *C. pneumoniae* was to increase CD25 expression.

CD25 is classified as a marker of cellular activation and is expressed on activated lymphocytes and monocytes (452-454). CD25 forms part of the receptor for IL-2, and expressed on its own is a low affinity receptor for IL-2 (192). However, the combination of CD25 (IL-2 receptor α chain) with CD122 (β chain) and CD132 (γ chain), forms a heterotrimeric IL-2 receptor complex with high affinity for its ligand IL-2 (192). A third combination of these molecules between CD122 and CD132 is also a receptor for IL-2, which exhibits intermediate affinity for IL-2 (192). Although CD25 alone binds IL-2, structural analysis shows that it has only a short intracellular portion and does not generate intracellular signal transduction pathways. The trimeric and dimeric IL-2 receptor complexes with the β and γ chains are able to generate signals important for T cell proliferation and growth (455).

Lymphocytes showed constitutive expression of CD25 on approximately 20% of unstimulated lymphocytes and was significantly increased to 35% after stimulation with PHA. Similar expression levels on T cells activated with different stimuli have also been reported (452,453). Although the changes in the percentage of lymphocytes expressing CD25 were statistically significant, the changes between uninfected cell lysate and *C. pneumoniae* stimulation only generated an extra 4% of cells expressing CD25. The changes in CD25 MFI between *C. pneumoniae* stimulation and uninfected cell lysate indicate an approximate 50% increase in the relative amount of protein expressed per cell. This suggests that although *C. pneumoniae* only caused minimal increases in the percentage of lymphocytes expressing CD25, the amount of protein expressed on those cells was significantly increased. Together with the percentage data, *C. pneumoniae* increased CD25 expression on lymphocytes but it is difficult to assess whether these changes are of biological significance without further studies.

Unstimulated lymphocytes do not express IL-2 receptors or IL-2, therefore, are unable to respond to exogenous IL-2 (454,456). Upon activation of T cells via the T cell receptor complex, transcription of IL-2 and IL-2 receptor genes are increased via activation of the phosphatidylinositol pathway (327,454). Transcription factors involved in IL-2 gene expression such as AP-1 and NF-AT are also increased by intracellular signals generated through CD69 ligation (442,443). Induction of CD25 expression on T lymphocytes via CD69 expression, however, which increases intracellular Ca^{2+}, requires simultaneous protein kinase C activation by phorbol esters such as PMA (328). CD69 crosslinking induces prolonged elevation of intracellular Ca^{2+}, which is required for protein kinase C activation (328,457). However, this signal is not sufficient to activate protein kinase C and therefore adequate CD25 expression is necessary for IL-2 mediated T cell proliferation (328). It is suggested that CD69 generates intracellular signals but simultaneous protein kinase C activation is required for fully functional activation of T cells. This may suggest that *C. pneumoniae* stimulates lymphocytes to a certain degree, possibly via the T cell receptor complex, inducing CD69 expression but minimal induction of CD25. Lymphocyte cytokine expression requires protein kinase C activation, which may partially explain the lack of *C. pneumoniae* induced lymphocyte cytokine expression. CD25 expression is inhibited in T cells by cyclic AMP but has no effect on CD69 expression (458). PGE_2 release by monocytes increases cyclic AMP in lymphocytes (433,437). *C. pneumoniae* may therefore, increase monocyte CD69 expression, generating PGE_2 release, increasing cyclic AMP in lymphocytes leading to inhibition of CD25 on lymphocytes. The CD25 promoter also contains a binding region for the transcription factor Stat1 which is inhibited by cyclic AMP (458,459). Further, Th2 cytokine patterns, which we were able to show in the previous chapter investigating *C. pneumoniae* modulation of monocyte cytokine expression, are also promoted by cyclic AMP (437).

In contrast to lymphocytes, we did not detect CD25 on the surface of unstimulated monocytes but it was significantly induced by overnight incubation with *E. coli* LPS and over 90% of

monocytes exhibited positive staining for CD25 as detected by flow cytometry. The low level of CD25 and induction by LPS on monocytes is consistent with previous publications from our laboratory and others (447,452,460,461). In addition, stimulation of whole blood with *C. pneumoniae* significantly increased CD25 expression on monocytes which was over and above the low level of expression induced by uninfected cell lysate. The percentage of monocytes positive for CD25 induced by *C. pneumoniae* was similar to that seen with *E. coli* LPS stimulation. These changes were also evident in the MFI data although *C. pneumoniae* stimulation did not increase the amount of CD25 protein per cell expressed on the monocyte surface to the same extent as *E. coli* LPS.

CD25 expression is induced on monocytes by IFN-γ (460,461). It is unlikely that endogenous IFN-γ is responsible for *C. pneumoniae* induced monocyte CD25 expression, as *C. pneumoniae* did not induce IFN-γ in whole blood lymphocytes (see Chapter 4). It is possible that *C. pneumoniae* LPS is responsible for the induction of CD25 as LPS from other bacteria also upregulate monocyte CD25 expression (447,452). *C. pneumoniae* may stimulate monocytes via Toll-like receptors. While Toll-like receptor 4 is predominantly a receptor for Gram negative bacterial LPS, chlamydial HSP-60 mediates its effects via the same receptor in vascular smooth muscle cells (412,462). The modulation of CD25 on monocytes however, may not be exclusively mediated via Toll-like receptor 4. Farina and colleagues have reported that ligands for Toll-like receptor 2, induce CD25 expression on monocytes (447). Furthermore, *C. pneumoniae* activation of peripheral blood mononuclear cells and monocyte derived dendritic cells to secrete cytokines is also dependent on Toll-like receptor 2 (249,425). In addition, the low levels of Toll-like receptors on neutrophils may explain the inability of *C. pneumoniae* to induce CD25 expression (364).

We did not detect CD25 on neutrophils under any circumstances. The lack of CD25 expression on neutrophils from unstimulated and stimulated cultures is consistent with the

findings of Herrmann and colleagues. They were unable to detect CD25 expression on resting granulocytes, or those incubated with IFN-γ, known to induce CD25 on monocytes (461).

It is well documented that CD25 expression is increased on T cells from asthmatics (135,137,237). T lymphocytes of asthmatics also show heightened responsiveness to viral pathogens by increasing CD25 in comparison to cells from control subjects (272). The relationship between CD25 expression and COPD is less well characterised with similar levels of expression between COPD patients and control subjects (301). The association between *C. pneumoniae* infection and chronic airways disease is well established. Positive serology indicative of acute or chronic infection occurs more often in asthmatics compared to controls and titres increase with disease severity (96,115,122). Our data may suggest that the activation status of lymphocytes seen in chronic airways disease, particularly CD25 expression, may in part be mediated by *C. pneumoniae* infection.

CD25 expression on monocytes in patients with chronic airways disease is not well characterised. However, monocytes play an active role in inflammatory processes and show elevated cytokine secretion in disease states. The IL-2 receptor also exists in a soluble form, the level of which is increased in plasma of asthmatics, compared to controls (429). The cell source of soluble IL-2 receptor could potentially be monocytes as well as T lymphocytes. As we have shown that *C. pneumoniae* increases surface expression of CD25 on monocytes, it is possible that *C. pneumoniae* may contribute to increased plasma levels of soluble IL-2 receptor in chronic airways disease.

The upregulation of CD69 and CD25 expression on monocytes, neutrophils and lymphocytes by *C. pneumoniae* has not been previously reported and suggest that all three cell types are activated to varying degrees after exposure to *C. pneumoniae* (**Figure 5.20**). Increased

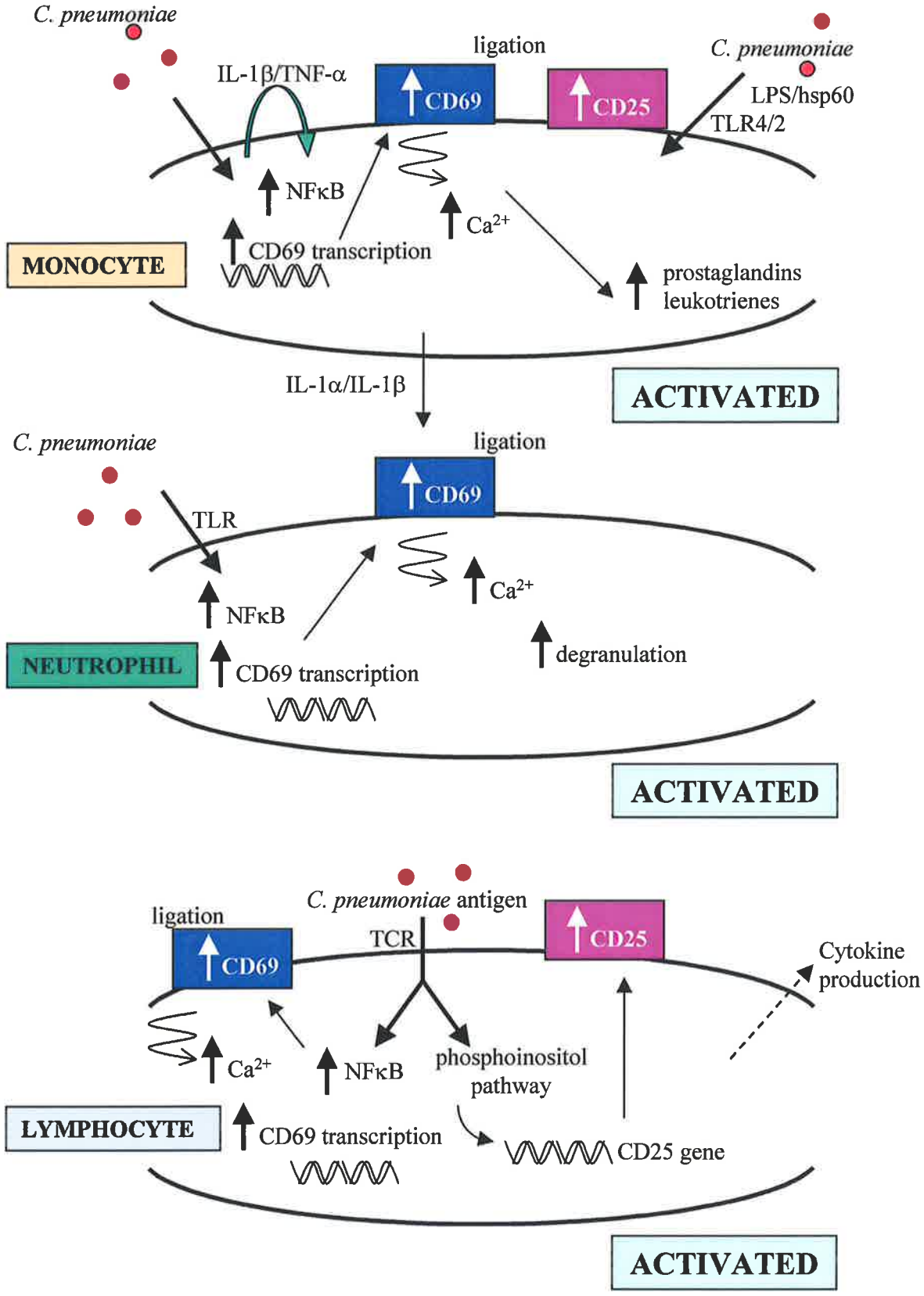


Figure 5.20: *C. pneumoniae* increases expression of activation molecules on leucocytes.
C. pneumoniae stimulation of whole blood causes increased CD69 expression on monocytes, neutrophils and lymphocytes and increased CD25 expression on monocytes and lymphocytes. The level of *C. pneumoniae* induced lymphocyte activation may not be sufficient however, to increase cytokine production. LPS, lipopolysaccharide; hsp, heat shock protein; TLR, Toll-like receptor. Ca^{2+}, intracellular calcium; TCR, T cell receptor.

expression of CD69 and CD25 on monocytes and neutrophils by *C. pneumoniae* may lead to optimal cellular activation and cytokine secretion. However, the overall immune response to the invading pathogen may be compromised by suboptimal activation of lymphocytes. Although our data in the current chapter suggests that lymphocytes are activated by *C. pneumoniae* with increased CD69 and CD25 expression, the level of this activation may not be sufficient to cause the downstream effects of cellular activation such as cytokine production which was not evident in the previous chapter. Minimal increases in the percentage and MFI of lymphocytes expressing CD25 after exposure to *C. pneumoniae* may be partly responsible for the failure of lymphocytes to secrete cytokines in response to this pathogen. Although *C. pneumoniae* appears to activate primary immune responses in monocytes and neutrophils, inability to clear infection due to dampened lymphocyte responses may allow development of persistent infection. *C. pneumoniae* infection of monocytes and neutrophils prolongs their survival (214,248,349) and together with suboptimal lymphocyte activation may provide an environment susceptible to the development of persistent infection.

After confirming that leucocytes are activated by *C. pneumoniae*, we next examined the expression of a panel of adhesion molecules after stimulation of leucocytes with *C. pneumoniae*. The effect of the IOL-207 *C. pneumoniae* isolate on leucocyte adhesion molecule expression has not been previously tested. We observed an increase in monocyte and neutrophil ICAM-1 expression after stimulation with *C. pneumoniae*. These results are in keeping with previously published studies using alternative *C. pneumoniae* strains causing increased ICAM-1 expression on peripheral blood monocytes and monocytic cell lines (252,255). The induction of ICAM-1 by *C. pneumoniae* has been reported on a variety of cells including airway epithelial cells and endothelium, (127,141,254). However, *C. pneumoniae* induced neutrophil ICAM-1 expression has not been previously described. Neutrophils are shown to express ICAM-1 and increase their expression in response to

TNF-α, GM-CSF and infection with *Staphylococcus aureus* (374). There are numerous papers reporting that ICAM-1 expression on respiratory epithelial cells is able to be modulated by TNF-α, IL-1β, IL-1α and IFN-γ (155,188,189,197). It is unlikely that endogenous IFN-γ is responsible for the increase in ICAM-1 positive neutrophils and monocytes stimulated with *C. pneumoniae*. In the previous chapter (Chapter 4), we did not observe IFN-γ production by *C. pneumoniae* stimulated lymphocytes. However, *C. pneumoniae* induced ICAM-1 expression on monocytes and neutrophils may be mediated by cytokines such as TNF-α and IL-1α, which we have shown to be increased by monocytes after *C. pneumoniae* stimulation (see Chapter 4).

The upregulation of ICAM-1 by *C. pneumoniae* may be mediated by a variety of intracellular signalling pathways. Activation of mitogen activated protein (MAP) kinase, protein kinase C and NFκB pathways are observed to be increased by *C. pneumoniae* infection of host cells, causing increased activity of transcription factors such as NFκB and AP-1 in cells including monocytes (141,422,463-465). These pathways are involved in the regulation of ICAM-1 expression (375) and may be activated via IL-1 and TNF-α (159,451,466). Therefore, *C. pneumoniae* stimulation of monocytes may increase the production of cytokines such as IL-1 and TNF-α which in turn, activates intracellular signalling pathways in monocytes and neutrophils resulting in upregulation of ICAM-1 transcription and eventually surface expression.

In contrast to monocytes and neutrophils, we detected only minimal ICAM-1 expression on lymphocytes. The level of ICAM-1 expression on resting and circulating lymphocytes is reported to be relatively low (309,313). Stimulation of cells with PHA resulted in a small but significant increase in the percentage of ICAM-1 positive lymphocytes but *C. pneumoniae* stimulation had no significant effects. A number of reasons may explain these results including the length of time whole blood cultures were stimulated. We stimulated cultures

overnight but other studies of T cell activation have shown that ICAM-1 expression is not maximally induced until 2-3 days of culture (315). This may also explain the modest increase in lymphocyte ICAM-1 expression after PHA stimulation.

The consequence of *C. pneumoniae* induced increased ICAM-1 expression on monocytes and neutrophils may allow these cells to interact with other inflammatory cells expressing CD11a, which is a ligand for ICAM-1. We therefore assessed CD11a expression on whole blood leucocytes in response to *C. pneumoniae* stimulation. The response of leucocytes to *C. pneumoniae* in terms of adhesion molecules, other than ICAM-1, has not been widely investigated. CD11a expression was decreased by *C. pneumoniae* on both monocytes and neutrophils but lymphocyte expression of CD11a was largely unaffected. CD11a combines with CD18 to form the β_2-integrin LFA-1 (192). In a study primarily investigating MHC molecule expression on U937 cells (a monocytic cell line) in response to *C. pneumoniae* infection, CD18 was also assessed. *C. pneumoniae* infection of U937 cells had no effect on CD18 expression but no further investigation of integrin expression was evaluated (252). There is some suggestion that decreased adhesion molecule expression, including CD11a by neutrophils, is due to the cells undergoing spontaneous apoptosis *in vitro*. (467). However, these studies also demonstrate that other adhesion molecules including CD58 and CD18 are maintained during neutrophil apoptosis (467,468). We also measured CD58 expression and observed a small but significant decrease in neutrophil expression, but no significant changes in monocyte CD58 expression. The conditions under which the current studies were performed favour the survival of neutrophils *in vitro*. Recent publications show that not only do neutrophils ingest *C. pneumoniae* but the bacteria remain viable and the survival of neutrophils is prolonged (349). Neutrophils infected with *C. pneumoniae* are shown to survive up to 90 hours *in vitro* (349). Further, the presence of monocytes in neutrophil cultures increases the survival of neutrophils (364). Neutrophil survival *in vitro* is also increased by LPS but this is also dependent on the presence of monocytes (364). Therefore,

in the current body of work, the use of whole blood, rather than isolated cells and the presence of *C. pneumoniae*, and chlamydial LPS may promote the survival of neutrophils. Therefore, the changes in adhesion molecule expression may be directly due to *C. pneumoniae* stimulation of neutrophils and not due to spontaneous apoptosis.

The decrease in CD11a on monocytes and neutrophils may alter the interaction of these cells with ICAM-1 positive inflammatory cells (neighbouring monocytes and neutrophils) and non-immune cells (epithelial cells). Adhesion between airway epithelial cells and eosinophils is mediated via the interaction between ICAM-1 and LFA-1 but only blocking antibodies to CD11a alter this interaction, in contrast to inhibition of ICAM-1, which has no effect (185). Similar interactions may occur between epithelium and neutrophils or monocytes via CD11a expression which is modulated by *C. pneumoniae* stimulation. Inhibition studies with blocking antibodies show that adhesion between *C. pneumoniae* infected monocytes and endothelial cells is mediated via β_2-integrins (469). However, the β_2-integrin family consist of various molecules, each member being composed of an alpha subunit (CD11a, CD11b, CD11c) and a β_2 subunit (CD18) (192). It is unclear which specific molecule(s) is inhibited in these studies and no further analysis of adhesion molecule expression was performed. Although the level of LFA-1 (CD11a/CD18) on circulating neutrophils is not significantly different between control subjects and patients with stable COPD, during exacerbations LFA-1 expression is significantly reduced (367). Acute *C. pneumoniae* infection is associated with COPD exacerbations (82,86,105). Therefore, changes in CD11a expression on neutrophils in exacerbations of chronic airways disease such as COPD may in part be due to *C. pneumoniae* infection.

The adhesion between monocytes or neutrophils and epithelial cells may be decreased due to decreased CD11a, despite maintenance of ICAM-1 on epithelium (see Chapter 3), resulting in an overall decreased physical association. Increased secretion of monocyte and neutrophil

chemoattractants such as IL-8 by *C. pneumoniae* infected airway epithelium, may facilitate migration of these cells to the initial site of infection. As monocytes and neutrophils are naturally phagocytic cells, initial interactions with epithelium may be strong until uptake of the bacteria occurs and subsequent decrease in CD11a mediated adhesion may allow migration of inflammatory cells away from the initial site of infection. *C. pneumoniae* may therefore play a role in promoting an environment that facilitates its transfer to susceptible inflammatory cells aiding systemic dissemination.

Lymphocytes appear to respond to *C. pneumoniae* to a lesser extent than monocytes and neutrophils. The expression of ICAM-1 on unstimulated lymphocytes was minimal and remained unchanged after *C. pneumoniae* stimulation. However, CD11a was expressed on the entire population of lymphocytes under all conditions tested and the MFI was not significantly changed by *C. pneumoniae* stimulation. This suggests that adhesion between monocytes or neutrophils and lymphocytes mediated via ICAM-1 and CD11a may overall be increased via upregulation of ICAM-1 expression on monocytes and neutrophils. Adhesion mediated via CD58 on neutrophils, and its ligand CD2, which is predominantly expressed on lymphocytes (192), overall, may not be significantly changed. The interaction may be maintained by decreased CD58 expression on *C. pneumoniae* stimulated neutrophils and increased CD2 expression on *C. pneumoniae* stimulated lymphocytes. In contrast, monocyte and lymphocyte adhesion may be increased due to maintenance of monocyte CD58 expression and the small but significant increase in CD2 expression on lymphocytes after exposure to *C. pneumoniae*. Ligation between CD2 and CD58 not only mediates adhesion but is also involved in costimulation of lymphocytes (319). CD2 ligation results in a variety of intracellular signals including activation of phosphoinositol pathways, increased intracellular Ca^{2+}, leading to T cell proliferation and cytokine production (318,319). These signals act in synergy with signals generated from the T cell receptor complex to increase T cell responses (311). Activation of the phosphoinositol pathway generates diacylglycerol

which is a potent activator of protein kinase C (470). Despite this understanding, due to the inability of lymphocytes to respond to *C. pneumoniae* to increase cytokine production, it still suggests that lymphocytes are not fully activated by the bacteria. The change in CD2 expression compared to uninfected cell lysate exposure was only a modest increase but prolongation of the stimulation is unlikely to cause a further increase in CD2 expression as activation of lymphocytes results in increased activity of the CD2/CD58 pathway within a period of hours (311).

The modulation of adhesion molecules on leucocytes by *C. pneumoniae* has not previously been comprehensively investigated. We have demonstrated that *C. pneumoniae* alters adhesion molecule expression predominantly on monocytes and neutrophils showing both cell specific and differential regulation (**Figure 5.21**). Monocytes and neutrophils increase ICAM-1 expression but lymphocytes are largely unaffected by *C. pneumoniae* exposure. In contrast neutrophils decrease their expression of both CD11a and CD58 whereas monocytes only show a reduction in CD11a expression. Lymphocytes maintain their expression of CD11a but show small increases in CD2 expression after *C. pneumoniae* stimulation. The modulation of adhesion molecules by *C. pneumoniae* may influence the interaction of inflammatory cells with both non- immune cells such as epithelial cells and their associations with inflammatory cells of different lineages. Modulation of these interactions may facilitate transfer of *C. pneumoniae* from the initial site of infection to disseminate throughout the body whilst maintaining an environment that promotes minimal activation of lymphocytes required to clear infection, possibly allowing the development of persistent infection.

Another process involved in the immune response and activation of T cells, is antigen presentation by professional antigen presenting cells. This process is mediated by the expression of MHC molecules by antigen presenting cells facilitating presentation of

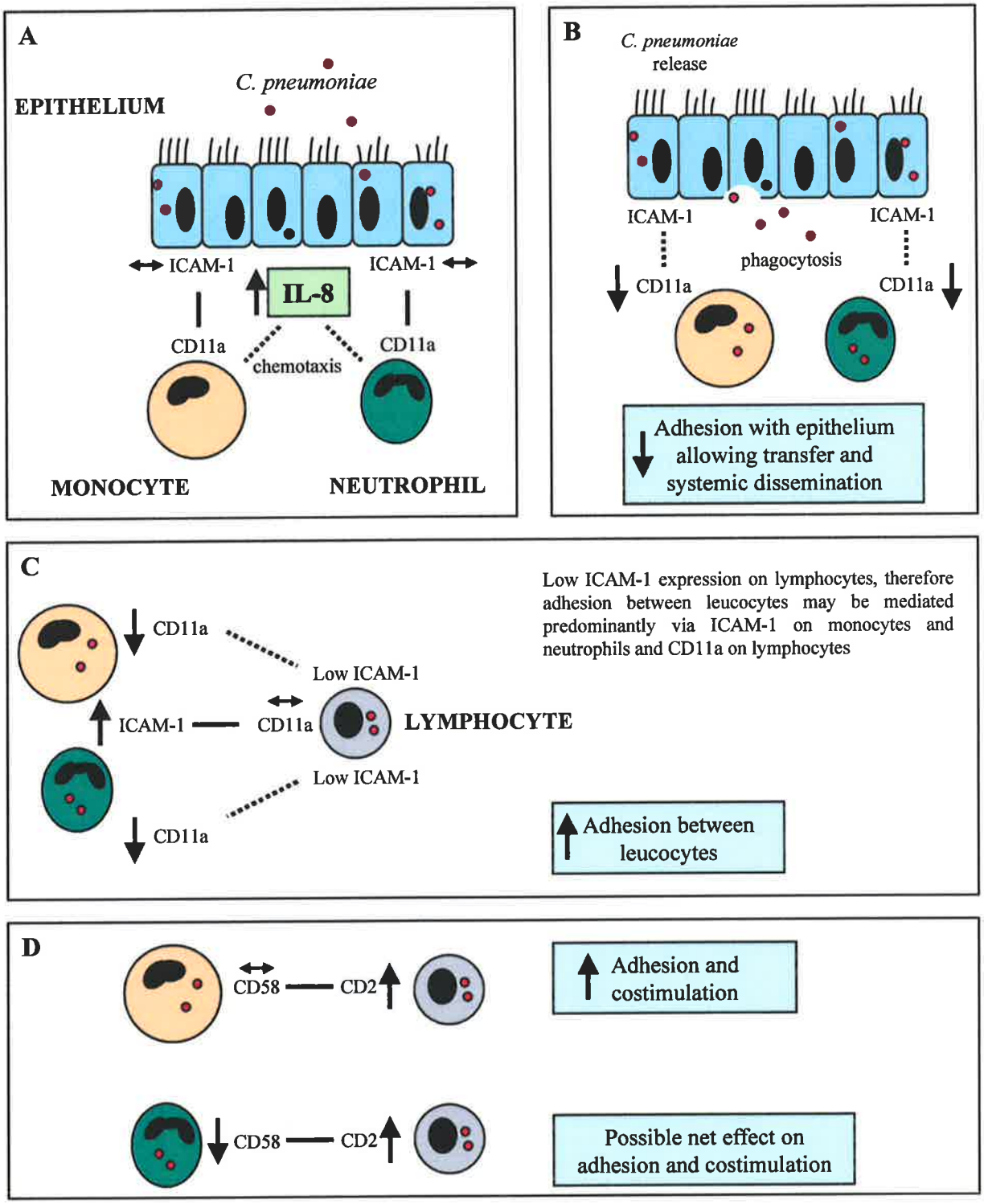


Figure 5.21: *C. pneumoniae* stimulation of whole blood leucocytes causes differential and cell specific modulation of surface markers involved in adhesion between immune and non immune cells.
A) *C. pneumoniae* infection of epithelium increases IL-8 expression, attracting monocytes and neutrophils to infection site. Adhesion is maintained by epithelial cell ICAM-1 expression (ligating with CD11a on leucocytes). **B)** *C. pneumoniae* is released by epithelial cells and phagocytosed by monocytes and neutrophils. Uptake of *C. pneumoniae* by monocytes and neutrophils causes decreased CD11a expression leading to decreased adhesion with epithelial cells. **C)** *C. pneumoniae* increases monocyte and neutrophil expression of ICAM-1, increasing adhesion mediated via CD11a lymphocyte expression. **D)** *C. pneumoniae* has no effect on monocyte CD58 expression but increases CD2 expression on monocytes causing an overall increase in adhesion and costimulation via this pathway. In contrast, the increase in lymphocyte CD2 expression but decrease in neutrophil CD58 expression, may result in no net change in activity of this pathway induced by *C. pneumoniae*.

processed antigen to lymphocytes. We therefore assessed the expression of MHC molecules on monocytes and neutrophils in response to *C. pneumoniae* stimulation. Although we consistently detected both MHC Class I and MHC Class I molecules on monocytes, and MHC Class I on neutrophils, the level of expression as indicated by the MFI, did not show a consistent pattern of expression after stimulation of cells with *C. pneumoniae*. After incubation of whole blood with uninfected cell lysate, both monocytes and neutrophils significantly changed their expression of MHC molecules. As these cells are professional antigen presenting cells, these changes may be due to the cells recognising the cellular debris as foreign material and could therefore be a non-specific response. In this case, the confounding effects and inconsistencies in the response to uninfected cell lysate incubation make it difficult to interpret the effect of *C. pneumoniae* stimulation on MHC molecule expression. This could be overcome by using a highly purified preparation of *C. pneumoniae*, reducing the amount of cellular debris in the bacterial preparation and therefore eliminating the requirement to use uninfected cell lysate as a negative control. It is well known amongst scientists within this field of research, that *C. pneumoniae* is a difficult bacteria to grow consistently in the laboratory. The protocols used in the current body of work are well established methods and are in line with previously published studies (68,144,357,464). Purification of *C. pneumoniae* by density gradient centrifugation requires large preparations of semi-purified *C. pneumoniae* and results in a significant decrease in bacterial yield. For these reasons, further purification of *C. pneumoniae* was not feasible for this study and semi-purified preparations were utilised.

Previous reports suggest that MHC Class I is downregulated on monocytes by *C. pneumoniae* via increased IL-10 expression but MHC Class II expression was not effected (252). We were able to demonstrate that *C. pneumoniae* stimulation increases the percentage of monocytes expressing IL-10 (see Chapter 4) but the induction with the IOL-207 isolate was not as efficient as the WA97001 isolate. As we used the IOL-207 isolate in the current studies

assessing surface marker expression, it is possible that the concentration of IL-10 induced by monocytes is not sufficient to significantly alter MHC Class I expression on monocytes. In contrast, alveolar macrophages and dendritic cells increase MHC Class II molecules after infection with *C. pneumoniae* (130,425). The data presented herein together with the above mentioned studies may suggest that *C. pneumoniae* modulation of MHC molecule expression is cell type specific.

Adequate T cell activation not only requires adhesion between antigen presenting cells and lymphocytes, and antigen presentation through MHC molecule expression, it also requires a second costimulatory signal. The main pathway of T cell costimulation is mediated via the interaction between CD28 on lymphocytes and the B7 family of costimulatory molecules. CD80 and CD86 are both part of the B7 family of cell surface molecules and bind to CD28 (471). These molecules are suggested to play a significant role in the differentiation of Th1 and Th2 cells and promotion of Th1 and Th2 patterns of cytokine expression (274,276,472). We therefore examined the role of *C. pneumoniae* in the modulation of leucocyte expression of these molecules.

CD80 expression was not detected on unstimulated monocytes but significantly increased and detected on 75% of monocytes after exposure to *E. coli* LPS. This data is consistent with previous studies documenting low levels of expression on resting monocytes and its upregulation by LPS (447). *C. pneumoniae* stimulation caused only a small increase in the number of monocytes exhibiting positive expression of CD80, up to 20% with the highest concentration used. However, incubation with uninfected cell lysate also induced CD80 expression but to a lesser extent than that seen with *E. coli* LPS. This may be a non-specific response of these professional phagocytes recognising cellular debris within the uninfected cell lysate preparation leading to cellular activation. Due to this result *C. pneumoniae* induction of CD80 expression on monocytes, must be compared to cells incubated with

uninfected cell lysate. Using this comparison we noted that CD80 expression on monocytes was significantly decreased when compared to cells incubated with uninfected cell lysate. Although CD80 expression has not been investigated in response to *C. pneumoniae* on monocytes, it is suggested in other systems to be downregulated by IL-10 (269). In the previous chapter we were able to induce low but significant IL-10 expression by whole blood monocytes stimulated with *C. pneumoniae* (see Chapter 4). In addition, ligands for Toll-like receptor 2 are shown to induce significant IL-10 expression in monocytes, but only low induction of CD80 in comparison to LPS induction of CD80 (447). Induction of CD80 by *E. coli* LPS may be a secondary effect of increased cytokine expression. Lipopolysaccharide is shown to increase GM-CSF by monocytes, and significantly increases CD80 expression on monocytes (223,447). This may suggest that monocyte CD80 expression is mediated via Toll-like receptors distinct from those activated by chlamydial LPS (Toll-like receptor 4) and mediated via alternative chlamydial antigens. Chlamydial HSP-60 is known to function via Toll-like receptor 4 but also generates intracellular signals via Toll-like receptor 2 (473). Other undefined chlamydial antigens, distinct from LPS are also shown to activate cells including monocytes via Toll-like receptor 2 dependent mechanisms (249,425). In addition, Toll-like receptor 2 activation of dendritic cells by *C. pneumoniae* causes increased translocation of NFκB to the nucleus (425). This suggests that *C. pneumoniae* may activate cells not only via LPS and Toll-like receptor 4 but also by alternative bacterial ligands for Toll-like receptor 2 and generate intracellular signalling pathways that lead to modulation of host cell transcription machinery.

In contrast to monocyte CD80 expression, CD86 was detected on approximately 70% of unstimulated monocytes and was subsequently decreased after exposure to *E. coli* LPS. Resting monocytes are reported to constitutively express CD86 (267,268,474). Farina and colleagues demonstrated a reduction in CD86 MFI on monocytes after overnight stimulation with LPS (447). The changes associated with *C. pneumoniae* stimulation were less clear in

terms of the percentage of monocytes expressing CD86. However, upon assessment of CD86 MFI, monocytes appeared to significantly decrease CD86 MFI after exposure to *C. pneumoniae*. A number of cytokines including TNF-α, IFN-γ and GM-CSF are shown to upregulate monocyte CD86 expression (273,447). As we detected increased TNF-α expression in *C. pneumoniae* stimulated monocytes, but decreased CD86 expression, it would suggest that the mechanism by which *C. pneumoniae* modulates CD86 is not mediated by endogenous cytokine secretion but by an alternative pathway. As with CD80 expression, this pathway may involve Toll-like receptors as various ligands for Toll-like receptors 4 and Toll-like receptor 2 caused a significant decrease in CD86 expression on monocytes (447).

CD28 is one of two ligands for CD80 and CD86, (the other being CTLA-4 (471)) and we assessed its expression on lymphocytes after exposure to *C. pneumoniae*. We demonstrated that CD28 is constitutively expressed on 80% of unstimulated lymphocytes which is consistent with published reports (335,340). There was a slight increase in lymphocyte CD28 MFI after PHA stimulation, confirming previous observations that the expression of this protein is inducible. However, *C. pneumoniae* stimulation did not significantly alter the percentage of lymphocytes expressing CD28 or the MFI of those that exhibited positive staining. Ligation of CD28 results in increased production of lymphocyte cytokines including TNF-α, IFN-γ, GM-CSF, IL-1, IL-2, IL-4 and IL-5 (338,339,472), thus regulating growth and proliferation. However, CD28 also plays a significant role in regulating the development of Th1 and Th2 differentiation. Ligation of CD80 and CD86 with CD28 promote Th1 and Th2 response patterns respectively (275,276,475). In chronic airways diseases such as asthma where the inflammatory pattern is described as a typical Th2 response with increased IL-4 and IL-5 expression, but low expression of IFN-γ, CD80 and CD86 expression are also reported to reflect an increase in Th2 responses. CD86 expression is upregulated on B cells of asthmatics but CD80 is decreased on macrophages (246,277). Other groups have reported that CD80 and CD86 are involved in both Th1 and Th2 responses (274,342,471,476). It is possible that Th1

responses required to clear infection are not adequately activated by *C. pneumoniae*, as current experiments show reduced expression of CD80. However, a corresponding increase in CD86 expression, promoting Th2 patterns, was not observed. In fact, *C. pneumoniae* caused a decrease in CD86 expression. Therefore, the effect of *C. pneumoniae* may be to decrease both Th1 and Th2 responses as part of a more generalised response of decreased activation of T lymphocytes. There are studies that show an overall decrease in costimulatory molecule expression in chronic airways disease with decreased CD80 and CD86 expression on peripheral blood mononuclear cells from asthmatics compared to control subjects (272). Furthermore, infection of cells with rhinovirus induces CD80 and CD86 expression, but is less marked in asthmatics (272). The response pattern and expression of CD80 and CD86 seen in COPD and asthma are similar to those that we have observed after direct stimulation of cells with *C. pneumoniae*. Given that a high percentage of these patients are now known to have evidence of chronic *C. pneumoniae* infection, the pattern seen could in part be mediated by *C. pneumoniae*. In addition, proliferation and activation of lymphocytes mediated via the CD28 pathway is shown to act in synergism with IL-12 (341). In the previous chapter (Chapter 4), we demonstrated that IL-12 expression by monocytes was decreased at higher concentrations of *C. pneumoniae*. Therefore, not only does *C. pneumoniae* reduce lymphocyte responses by reducing the costimulatory signals provided by monocyte expression of CD80 and CD86, it may also decrease signals that synergise with this pathway and are summarised in **Figure 5.22**.

Another pathway involved in the costimulation of lymphocytes is the interaction between CD40 on antigen presenting cells and CD40L on lymphocytes. We therefore assessed *C. pneumoniae* modulation of this pathway by measuring CD40 on monocytes and CD40L on lymphocytes. Unstimulated monocytes did not express CD40 but it could be induced by *E. coli* LPS stimulation. These observations are consistent with previous publications showing low expression of CD40 on unstimulated monocytes but significant induction after

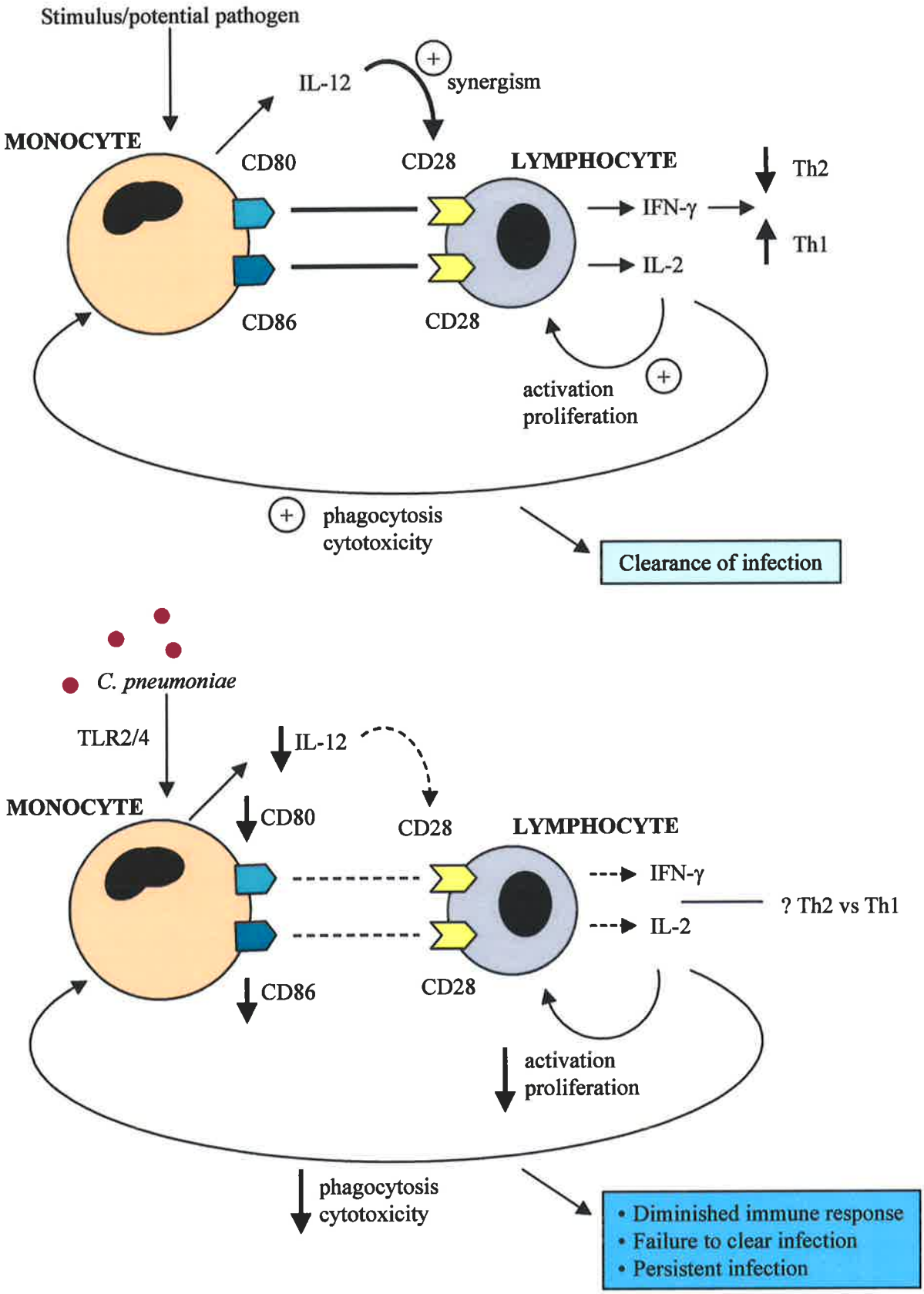


Figure 5.22: *C. pneumoniae* reduces costimulatory molecule expression on monocytes.
C. pneumoniae stimulation of whole blood monocytes decreases CD80 and CD86 expression which may result in inadequate lymphocyte activation via the CD28 pathway. Reduced lymphocyte activation and proliferation may lead to a failure to clear *C. pneumoniae* infection and allow the development of persistence.

stimulation (260,261). In addition, at the highest concentration of *C. pneumoniae*, monocytes were induced to express CD40 on approximately 75% of the cell population. Dendritic cells are also stimulated by *C. pneumoniae* to increase CD40 expression via NFκB stimulation (425) but no studies of monocyte CD40 modulation by *C. pneumoniae* have been reported. It is possible that the upregulation of CD40 on monocytes is also mediated by increased NFκB activity as this has been shown to occur upon *C. pneumoniae* infection of monocytes (422). Dendritic cell stimulation by *C. pneumoniae* and increased NFκB activity leading to increased CD40 expression is shown to occur via a Toll-like receptor 2 dependent mechanism, independent of Toll-like receptor 4 suggesting that chlamydial LPS may not play a role in CD40 induction. However, other Gram negative endotoxins are suggested to be recognised by Toll-like receptor 4 as well as additional Toll-like receptors (477-479). Monocytes stimulated with various cytokines including IFN-γ, GM-CSF and IL-3 are shown to increase CD40 expression (260,261). However, as previously mentioned, lymphocyte cytokines such as IFN-γ and IL-3 are unlikely to be responsible for induction of surface markers on neighbouring cells in this system as we did not detect any cytokine production from lymphocytes after *C. pneumoniae* stimulation (see Chapter 4).

The ligand for CD40 is CD40 ligand (CD40L) which is expressed on activated T cells, B cells, basophils, eosinophils and monocytes (480,481). We assessed CD40L expression on lymphocytes after *C. pneumoniae* stimulation but did not detect CD40L under any of the conditions tested. Together with the data already presented within this chapter, this provides further evidence that lymphocytes are largely unresponsive to direct stimulation by *C. pneumoniae*. Despite this, the expression of CD40L on other cell types may influence monocyte activation by interaction with increased CD40 expression after *C. pneumoniae* infection. Ligation between these two molecules on monocytes induces the production of cytokines including IL-1β, IL-1α, TNF-α, IL-6, and IL-8 (261,480). Increased ICAM-1 expression on monocytes may also be due to CD40 and CD40L interactions (261).

The main findings in this chapter of work indicate that *C. pneumoniae* modulates the expression of a variety of surface markers on whole blood leucocytes. These changes are predominantly reflected on monocytes and neutrophils with only minimal changes to lymphocyte surface marker expression. The modulation of adhesion molecules on neutrophils and monocytes may initially promote transfer and dissemination of *C. pneumoniae* from the epithelium to systemic sites of infection. Although monocytes and neutrophils possess cytotoxic machinery for defence against invading organisms, *C. pneumoniae* is capable of surviving in these cells, increasing the likelihood of developing into a persistent infection. Recently, van Zandbergen and colleagues showed that neutrophils not only internalise *C. pneumoniae*, but the bacteria survive and multiply within these cells (349). This suggests that there may be a disruption to the regular host cell processes required to kill the bacteria. In contrast to other invading micro-organisms that induce host cell apoptosis (482,483), *C. pneumoniae* prolongs neutrophil survival by delaying apoptosis (349). Uninfected neutrophils underwent apoptosis and survived for half the time of *C. pneumoniae* infected cells. Similarly, monocytes infected with *C. pneumoniae* are also shown to be resistant to apoptosis and is dependent on NFκB activity and IL-10 secretion (248,422). *C. pneumoniae* is however, capable of replicating within these cells and can be grown *in vitro* for up to two weeks (215). Therefore, *C. pneumoniae* may promote the development of persistent infection by increasing adhesion between leucocytes, allowing transfer and dissemination of the organism and extending the lifespan of host cells such as monocytes and neutrophils.

Adhesion between monocytes and neutrophils with lymphocytes may be modulated by *C. pneumoniae* to facilitate antigen presentation. The association of typical adhesion molecules on monocytes and neutrophils with their ligand counterparts on lymphocytes may also play a role in the provision of a costimulatory signal. However, the overall balance of costimulatory signals provided to lymphocytes may be decreased by reduced CD80 and CD86

expression on monocytes. Inadequate costimulation of lymphocytes via the CD28 pathway may be a mechanism by which *C. pneumoniae* escapes detection by the immune system.

In comparison to other pathogens, whether bacterial or viral, *C. pneumoniae* may elicit host cell responses that are unique to this micro-organism and are important in regulating lymphocyte costimulation and ultimately cell mediated immunity. Clearance of infection with other intracellular pathogens such as *Mycobacterium tuberculosis* also requires efficient lymphocyte responses (484). However, *Mycobacterium tuberculosis* has also evolved strategies that culminate in the development of persistent infection, some of which involve cell surface molecule expression. Infection of macrophages with *Mycobacterium tuberculosis* increases ICAM-1 and LFA-1 (CD11a/CD18) expression, decreases antigen presentation and costimulatory molecules (MHC Class II and B7 molecules) contributing to reduced cell mediated immunity (485-487). We did not observed consistent changes in MHC Class II molecules on any cell type and although detected increased ICAM-1 on monocytes and neutrophils, CD11a expression was significantly reduced on both monocytes and neutrophils. Therefore, in comparison to *Mycobacterium tuberculosis*, the data presented in the current studies suggests that *C. pneumoniae* may predominantly affect the CD28 costimulatory pathway via reduced monocyte expression of the B7 family of molecules (CD80 and CD86). Decreased CD11a may reduce interactions between antigen presenting cells, also decreasing the likelihood of costimulatory signal delivery.

It is difficult to directly compare the response of monocytes to *E. coli* LPS stimulation, as the concentration of chlamydial LPS in the *C. pneumoniae* preparation is unknown and the potency of endotoxin differs between pathogens (36,40). However, induction of monocyte CD80 expression by *C. pneumoniae* was relatively low in comparison to *E. coli* LPS stimulation. Whereas, the pattern of monocyte CD86 expression was similar between the two treatments.

Not surprisingly, the response of monocytes to *C. pneumoniae* bacterial infection appears to be different to that seen with viral infection. Rhinovirus infection of monocytes causes significant increase in the percentage of CD14 positive cells expressing CD80, (up to 70%) in comparison to control cultures consisting of uninfected cell lysate (272). This data is in contrast to the results presented herein, where although *C. pneumoniae* infection of monocytes caused an increase in CD80 expression compared to baseline unstimulated cells to 20%, in comparison to uninfected cell lysate treated cells, a significant decrease in CD80 expression was observed. When analysis of monocyte CD86 expression is compared between *C. pneumoniae* and rhinovirus, both pathogens cause a decrease in CD86 MFI in comparison to control cells (272). Similar to our results with *C. pneumoniae*, CD28 expression on lymphocytes was not modulated by rhinovirus which may suggest that the effects of invading micro-organisms are mediated predominantly on antigen presenting cells and may be different to that observed after viral infection.

It has become evident in recent years that persistent *C. pneumoniae* infection is associated with chronic airways disease and inflammation. As the airway epithelium is the primary site of *C. pneumoniae* infection, the infiltration of leucocytes into the airways in asthma and COPD increases the likelihood of these cells becoming infected as a result of release of infectious *C. pneumoniae* progeny from the epithelium. In the current chapter we have extended our understand of the host cell-pathogen relationship by examining the response of leucocytes to *C. pneumoniae* in terms of surface marker expression. We have shown that *C. pneumoniae* activates a range of host cells, as evidenced by CD25 and CD69 expression on whole blood leucocytes, which has not previously been reported. However, the extent of cellular activation, with particular emphasis of lymphocyte activation, requires further study. The expression pattern of activation molecules on peripheral blood leucocytes in response to direct stimulation with *C. pneumoniae* is similar to that seen in asthma and COPD. This may

suggest that in patients with chronic respiratory disease the activation of leucocytes may in part be due to infection with *C. pneumoniae*. In addition, we have shown that *C. pneumoniae* modulates expression of adhesion molecules in a cell type specific manner, predominantly affecting monocytes and neutrophils. Once infection has been established in leucocytes, as a result of airway epithelial cell lysis, reduced adhesion between epithelial cells and leucocytes may provide an opportunity for *C. pneumoniae* to disseminate systemically. The survival of *C. pneumoniae* within leucocytes is promoted by interruption to host cell processes including apoptotic pathways. *C. pneumoniae* infection of leucocytes may also alter surface expression of molecules involved in adhesion between leucocytes. However, surface markers classically described as adhesion molecules are also involved in other cellular processes including costimulation, therefore, the ability of *C. pneumoniae* to modulate adhesion molecules may alter the capability of the immune system to fight infection at multiple levels. Adhesion molecules also facilitate antigen presentation by maintaining close proximity of antigen presenting cells and lymphocytes. The expression of MHC molecules involved in antigen presentation was largely unchanged by *C. pneumoniae* in the current studies but coupled with reduced capacity of monocytes to provide costimulatory signals may be of great importance. The reduced expression of costimulatory molecules induced by *C. pneumoniae* may be a way in which this unique bacteria evades the immune system facilitating the development of persistent infection. Overall our studies have highlighted the diversity of the host cell-pathogen relationship with regard to surface molecule expression by leucocytes in response to *C. pneumoniae* exposure. The modulation of adhesion and costimulatory pathways in leucocytes may be paramount in understanding how the development of persistent *C. pneumoniae* infection is initiated in chronic airways disease states.

CHAPTER 6

RESULTS

Modulation of surface marker expression profiles of peripheral blood leucocytes by *C. pneumoniae* infected airway epithelial cells

Introduction

In the preceding chapters we have shown that epithelial cells and leucocytes derived from whole blood are susceptible to *C. pneumoniae* infection. In the current chapter we explore more directly the interactions between infected airway epithelial cells and leukocytes. Airway epithelium is the primary site of *C. pneumoniae* infection within the respiratory tract (128,146,357). In animal models of *C. pneumoniae* infection, the resultant inflammatory infiltrates consist of both neutrophils and monocytes (146,357). Further, infection of epithelium and endothelium with *C. pneumoniae* enhances neutrophil and monocyte transmigration, indicating that these cells play an active role in the recruitment of leucocytes to the infection site (127,356,377). The chemotaxis of leucocytes to the site of *C. pneumoniae* infection is mediated in part by IL-8 and MCP-1 release from infected cells (356,377). We have shown that *C. pneumoniae* infection of airway epithelial cell lines induces the production of IL-8 (see Chapter 3), which is a potent chemotactic factor for neutrophils (219). In addition, adhesion molecules on both epithelium and leucocytes may also be involved in the chemotaxis process. Adhesion between *C. pneumoniae* infected endothelial cells and monocytes and neutrophils is mediated via upregulation of endothelial surface expression of ICAM-1 and VCAM-1 and blocked by antibodies to β_2 integrins including CD11a/CD18 and C11b/CD18 (141). Adhesion between leucocytes and endothelium is also mediated via leucocyte CD62L (L-selectin) expression (361,488). Limited studies have been performed examining *C. pneumoniae* infection of airway epithelial cells and the role of surface markers involved in adhesion between leucocytes and epithelial cells. However, transepithelial neutrophil migration induced by *C. pneumoniae* infected

airway epithelial cells is also shown to involve epithelial expression of ICAM-1 (127). We have previously examined the direct effect of *C. pneumoniae* infection of leucocytes and examined their response in terms of cytokine and surface marker expression. Lysis of host cells (airway epithelium) would potentially release infectious *C. pneumoniae* progeny enabling infection of neighbouring cells and infiltrating inflammatory cells, modulating their expression of both surface molecules and cytokines. Additionally, we were also interested in the relationship between *C. pneumoniae* infected airway epithelial cells and the impact that they may have (via secreted cytokines or other factors) on neighbouring and infiltrating cells during the infection, before host cell lysis.

During *C. pneumoniae* infection of bronchial epithelial cells we were able to detect IL-8 and IL-6 (see Chapter 3). *C. pneumoniae* infection of airway epithelium may also cause the release of other factors including GM-CSF, MCP-1 or bacterial products which are known to be released from *C. pneumoniae* infected endothelial cells, cervical epithelial cells and colonic epithelial cells (391,402,473). The epithelium is a rich source of these factors. In addition, in chronic airways diseases, the levels of IL-8, IL-6 and GM-CSF are increased (137,165,167,168,180). These cytokines are involved in the regulation of leucocyte cellular functions including chemotaxis, phagocytosis and adhesion (356,369,489). In order for infiltrating leucocytes to function efficiently and combat infection they must be under strict regulation. A variety of processes utilised to effectively clear infection such as phagocytosis, chemotaxis and respiratory burst in neutrophils involve adhesive mechanisms (358). As previously described cellular adhesion is mediated by a variety of cell surface molecules (see **Table 5.1**). One of the key molecules involved in neutrophil defence mechanisms is the β_2 integrin, CD11b/CD18. It is also expressed on monocytes and is involved in phagocytosis and the generation of reactive oxygen species (489).

Adhesion between epithelial cells and leucocytes may provide additional information regarding inflammation in chronic airways disease. In chronic airways disease significant damage to the respiratory epithelial lining is observed, often inflicted by inflammatory cells such as neutrophils and eosinophils (185,367,490). Adhesion of neutrophils to airway epithelium has the potential to exacerbate inflammation if the activation and function of these leucocytes is not strictly regulated (361). The airway epithelium serves as a first line of defence in the fight against infection. It is ideally located to respond to potential pathogens and plays an active role in the recruitment and regulation of inflammatory cells. *C. pneumoniae* infection of airway epithelium may contribute to the regulation of inflammatory cell activation and immune responses.

Aims

The overall aim of the studies described in this chapter was to investigate the host-pathogen relationship by investigating the surface marker expression profile of leucocytes after exposure to conditioned media from *C. pneumoniae* stimulated airway epithelial cells.

The specific aims were:

a) To investigate whether *C. pneumoniae* infected airway epithelial cells activate monocytes.

b) To investigate whether *C. pneumoniae* infected airway epithelial cells activate neutrophils.

c) To determine whether monocytes alter adhesion molecule expression after exposure to conditioned media from *C. pneumoniae* infected airway epithelial cells.

d) To determine whether neutrophils alter adhesion molecule expression after exposure to conditioned media from *C. pneumoniae* infected airway epithelial cells.

Methods

C. pneumoniae was propagated in HEp-2 cells, and harvested according to the protocol detailed in the methods chapter (Chapter 2). Uninfected cell lysate was also prepared in the same manner from uninfected HEp-2 cells.

The human bronchial epithelial cell line, BEAS-2B was maintained as described in Chapter 2. In order to obtain conditioned media from BEAS-2B cells stimulated by *C. pneumoniae*, cells were plated at $3x10^5$ cells/ml into 24 well plates. After allowing the cells to adhere overnight, medium was replaced with fresh medium with or without stimulus in a total volume of 1ml. Stimulus consisted of *C. pneumoniae* (IOL-207 or WA97001) or a comparable amount of uninfected cell lysate (which served as a negative control). As a positive control for IL-8 production, cells were stimulated with TNF-α (20ng/ml). A corresponding negative control consisted of cells to which no additional stimulus was added. Cells were incubated for 24 hours before removing media (stored at -70°C) and washing the cells three times with 1 X PBS to remove any extracellular *C. pneumoniae* organisms which may have attached to the cells. Medium was then replaced with fresh medium and incubated for a further 24 hours. At this time, conditioned medium was collected and frozen at -70°C until required.

As a control for this study, a second 24 well plate was prepared in a similar manner without BEAS-2B cells. These treatments were set up to account for any carry over of *C. pneumoniae*, or proteins present in uninfected cell lysate or cell culture media, which may adhere to the cell culture plates and that may act as a stimulus.

Conditioned media samples collected from BEAS-2B cells stimulated with *C. pneumoniae* or media in the absence of cells, was assayed for IL-8 content by ELISA, as described in Chapter 2. Measurement of IL-8 release into cell supernatant served as an internal experimental control, ensuring that the cells were in an active state and responsive to stimuli.

In order to assess whether *C. pneumoniae* stimulates epithelial cells to secrete products that act on downstream inflammatory processes, we analysed the response of whole blood leucocytes to conditioned media from epithelial cells by flow cytometry. Venous blood was collected into tubes containing 20 units/ml sodium heparin as an anticoagulant, from volunteers, after obtaining informed consent, who were non atopic, non smokers with no history of respiratory disease.

A single serum sample from each volunteer was also obtained to test for the presence of *C. pneumoniae* specific IgG and IgM antibodies using the MIF test (see Chapter 2) to establish whether volunteers had previously been exposed to the bacteria.

The remaining whole blood was diluted 1:1 with conditioned media from BEAS-2B cells as described above and incubated overnight. Another set of tubes were set up consisting of whole blood diluted 1:1 in serum free cell culture medium and incubated overnight with *C. pneumoniae*, a comparable concentration of uninfected cell lysate (which served as a negative control) or *E. coli* LPS (100ng/ml; which served as a positive control for monocyte stimulation), or cells were incubated in media alone, serving as an unstimulated control. These treatments were used as a positive control for stimulation of whole blood leucocytes.

The expression of cell surface molecules was detected and measured using flow cytometry as described in Chapter 2 and shown in **Figure 2.13**. Whole blood monocytes were gated based on $CD14^+$ staining and side scatter characteristics (see **Figure 2.14**). Whole blood neutrophils were gated based on forward and side scatter characteristics (see **Figure 2.15**). These cells were then formatted in dot plots displaying positive staining for cell surface molecules involved in adhesion and activation processes. The relative amount of protein expressed on the cell surface was determined by selecting gated cells (monocytes or neutrophils) and

formatting these cells in histograms displaying the mean fluorescent intensity of cells staining positively for the cell surface molecule of interest (see **Figure 2.14 – 2.15**). Cell surface molecules measured in this chapter of work included markers of cellular activation (CD25 and CD69) and adhesion molecules (CD11b and CD62L). To check the purity of the neutrophil population of cells, CD16 was used to discriminate between neutrophils and eosinophils.

Results

To confirm that the BEAS-2B cells were active and responsive to stimuli, conditioned medium obtained from BEAS-2B cells stimulated overnight with *C. pneumoniae* was assayed for the presence of IL-8 protein by ELISA. BEAS-2B cells increased their production of IL-8 in response to *C. pneumoniae*, with the WA97001 isolate being more potent than IOL-207 (**Figure 6.1**). These results were consistent with those described previously in Chapter 3. Wells which did not contain BEAS-2B cells but were incubated overnight with media, with or without stimuli, did not contain any detectable levels of IL-8 protein (**Figure 6.1**).

After 24 hours the BEAS-2B cells were washed then incubated with fresh media for a further 24 hours. This latter medium was then collected, analysed and shown to contain IL-8 which was generally at a higher concentration than that detected in medium from the first 24 hour incubation period (**Figure 6.1**) except for the highest dose of WA97001, where the greater potency of this agent may have lead to an earlier peak in IL8 production. Similar to the first incubation period, wells not containing BEAS-2B cells did not contain any significant levels of IL-8. These results indicated that the BEAS-2B cells were responsive to stimuli and that IL-8 protein was still present in cell culture media up to 48 hours post stimulation with *C. pneumoniae*.

Our next aim was to determine whether the conditioned media obtained from *C. pneumoniae* infected BEAS-2B cells could modulate the expression of leucocyte surface molecules. We collected blood from healthy volunteers and incubated it overnight with conditioned media from *C. pneumoniae* infected BEAS-2B cells.

To determine whether volunteers had previously been exposed to *C. pneumoniae*, serum from each blood sample was analysed for *C. pneumoniae* specific antibodies using the MIF test. All serum samples were positive for *C. pneumoniae* specific IgG antibodies analysed at a

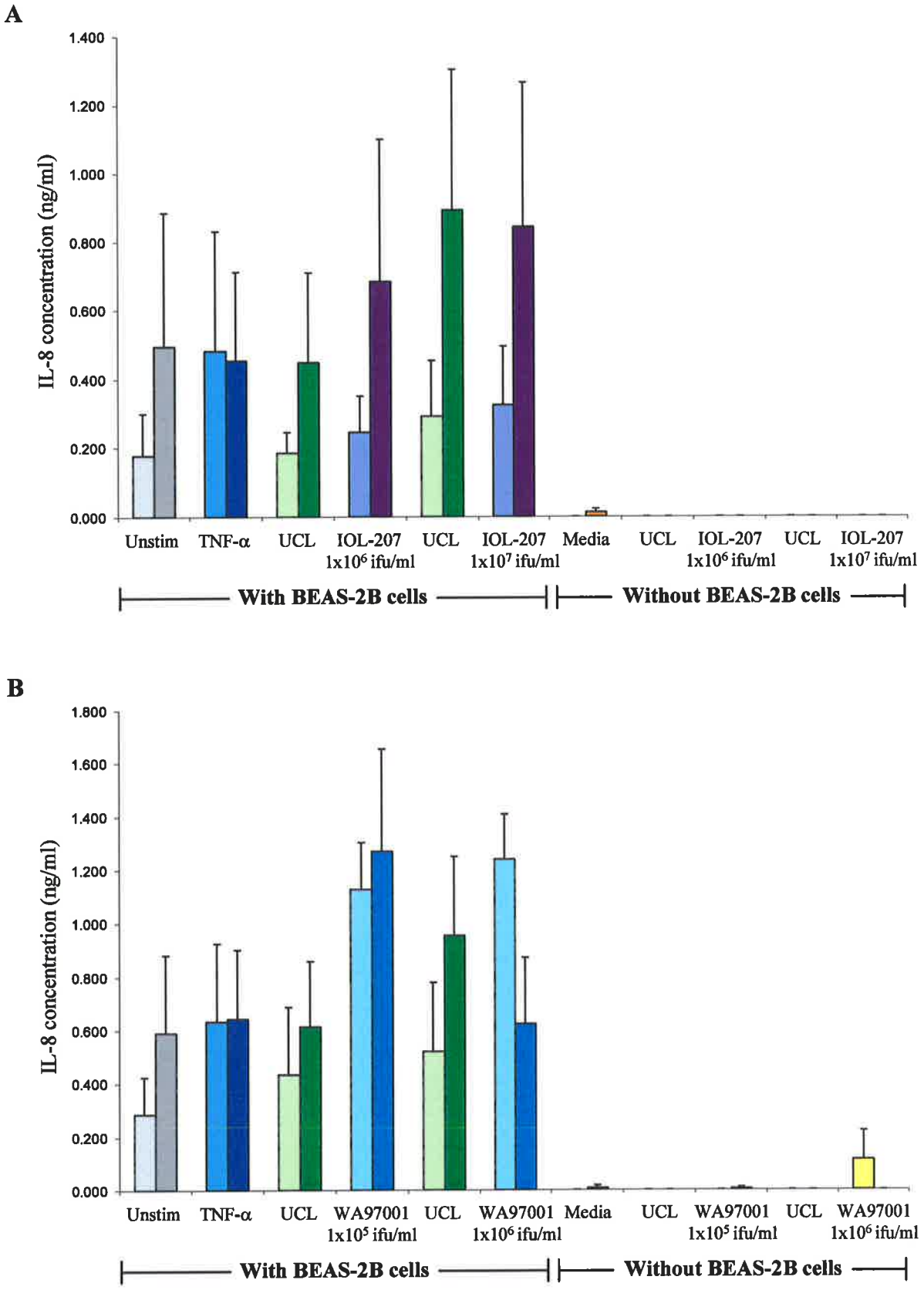
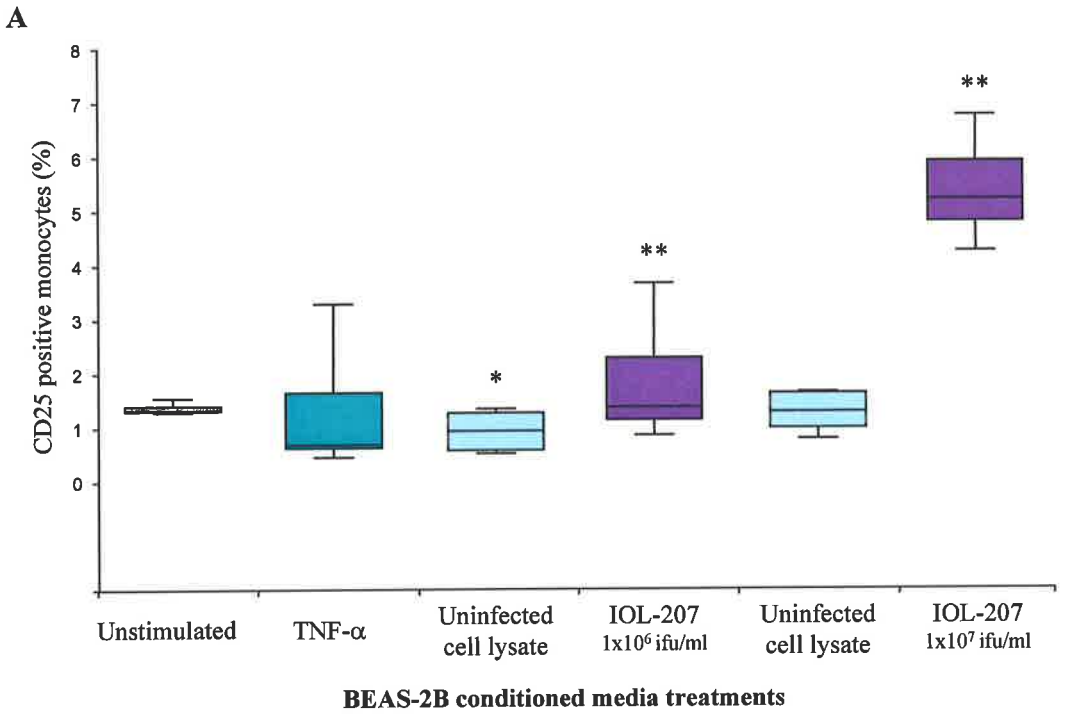


Figure 6.1: IL-8 production by *C. pneumoniae* infected BEAS-2B cells.
Cells were plated at $3x10^5$ cells/ml and incubated overnight with media alone, TNF-α, uninfected cell lysate or *C. pneumoniae*. A second set of wells were prepared in the same manner, without BEAS-2B cells. The following day media was collected and assayed for IL-8 by ELISA, light coloured bars. Cells (and wells) were washed 3X with PBS before replacing with fresh cell media and plates incubated for a further 24 hours. Media was collected and assayed for IL-8 by ELISA, dark coloured bars. Graphs represent mean ± SEM of **A)** BEAS-2B cells infected with IOL-207, 3 separate experiments or **B)** WA97001 *C. pneumoniae* isolate, 4 separate experiments, performed in triplicate.

single dilution of 1:16. The criteria used in the current study to identify individuals with pre-existing antibody or evidence of past infection is in accordance with previously defined criteria (3,84,88). These studies have defined past infection as a serum sample with an IgG titre ≥1:16 and <1:512. The results obtained from the MIF test indicate that all subjects participating in this study had evidence of past infection with *C. pneumoniae*.

Are monocytes activated by C. pneumoniae infected airway epithelial cells?

In order to assess whether *C. pneumoniae* stimulated epithelial cells to secrete products which may activate whole blood leucocytes, conditioned media from BEAS-2B cells were incubated with whole blood overnight. To assess activation of whole blood monocytes, we used flow cytometry to measure the expression of CD25 and CD69. Less than 2% of whole blood monocytes incubated with conditioned media from unstimulated BEAS-2B cells expressed CD25 (**Figure 6.2**). Conditioned media from BEAS-2B cells stimulated with TNF-α or incubated with uninfected cell lysate did not significantly increase the percentage of monocytes expressing CD25. However, media obtained from BEAS-2B cells stimulated with $1x10^7$ or $1x10^6$ ifu/ml of *C. pneumoniae* (IOL-207) significantly increased CD25 expression on monocytes up to 5% compared to appropriate amount of uninfected cell lysate (**Figure 6.2A**). Blood incubated with media from BEAS-2B cells infected with the WA97001 isolate also showed a trend toward increased monocyte CD25 expression but it did not reach statistical significance (**Figure 6.2B**). Analysis of monocyte CD25 MFI showed that medium from chlamydia-infected BEAS-2B cells had no effect on this parameter (**Table 6.1A and B**).



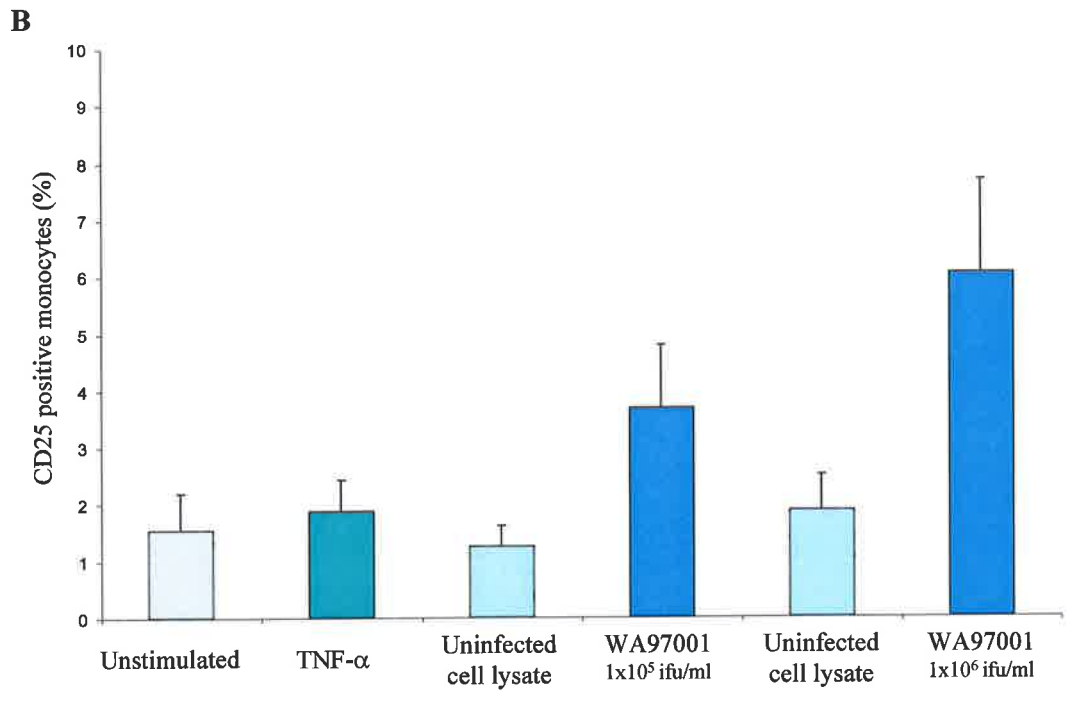


Figure 6.2: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells increases monocyte CD25 expression.

BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and monocyte CD25 expression detected using a CD25 FITC monoclonal antibody and measured by flow cytometry. **A)** Boxes represent the interquartile range of the percentage of monocytes expressing CD25 after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* (IOL-207) from 2 separate experiments performed in triplicate on whole blood from 2 different volunteers. Whiskers represent the highest and lowest values, with the line across the box representing the median. The reciprocal square root of the raw data was calculated before applying the mixed ANOVA model for analysis. **B)** Bars represent the mean ± SEM of the percentage of monocytes expressing CD25 after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* (WA97001) from 3 separate experiments performed in triplicate on whole blood obtained from 3 different volunteers. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of uninfected cell lysate, mixed ANOVA.

Mean fluorescent intensity of monocytes expressing CD25						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	126.08	57.41	55.92	50.39	61.02	52.26
2	44.44	39.10	40.20	47.02	37.45	48.37
Mean	85.26	48.26	48.06	48.71	49.23	50.32
SEM	40.82	9.16	7.86	1.68	11.78	1.94

Table 6.1A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not affect the MFI of monocytes expressing CD25.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Monocytes were gated based on $CD14^+$ staining by flow cytometry and CD25 MFI was determined using an anti-human FITC labelled monoclonal CD25 antibody. Data represents the mean MFI of monocytes expressing CD25 from 2 individual subjects, performed in triplicate.

Mean fluorescent intensity of monocytes expressing CD25						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	126.08	57.41	55.92	66.01	61.02	63.64
2	53.64	59.18	57.53	54.65	52.41	50.27
3	60.10	70.85	61.94	72.12	67.71	70.45
Mean	79.94	62.48	58.46	64.26	60.38	61.45
SEM	23.14	4.22	1.80	5.12	4.43	5.93

Table 6.1B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect the MFI of monocytes expressing CD25.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Monocytes were gated based on $CD14^+$ staining by flow cytometry and CD25 MFI was determined using an anti-human FITC labelled monoclonal CD25 antibody. Data represents the mean MFI of monocytes expressing CD25 from 2 individual subjects, performed in triplicate.

Media obtained from wells incubated with the desired stimulus in the absence of BEAS-2B cells had no significant effect on the percentage of monocytes expressing CD25 and was expressed on less than 4% of monocytes under all conditions.

As we had shown that *C. pneumoniae* infection of BEAS-2B cells stimulated IL-8 production, we investigated whether IL-8 was responsible for increased monocyte CD25 expression. However, direct stimulation of whole blood monocytes with IL-8 did not significantly alter

the percentage of monocytes expressing CD25 compared to unstimulated cells, although a response was seen to the *E. coli* LPS positive control (**Table 6.2**).

Percentage of monocytes expressing CD25			
Subject	**Unstimulated**	***E. coli* LPS**	**IL-8**
1	2.40	60.30	1.42
2	1.23	70.62	1.78
3	1.57	76.17	2.07
Mean	1.73	69.03*	1.76
SEM	0.35	4.65	0.19

Table 6.2: Direct stimulation of whole blood with IL-8 does not stimulate monocyte CD25 expression. Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), or IL-8 (5ng/ml). Monocytes were gated based on $CD14^+$ staining by flow cytometry and CD25 positive monocytes detected using an anti-human FITC labelled monoclonal CD25 antibody. Data represents the mean percentage of monocytes expressing CD25 from 3 individual subjects, performed in triplicate. * $p<0.05$ compared to unstimulated, mixed ANOVA.

As a second indicator of cellular activation, the expression of CD69 was measured on monocytes after incubation with conditioned media from *C. pneumoniae* infected airway epithelial cells. There were approximately 40-60% of monocytes which were positive for CD69 expression after incubation with conditioned media from unstimulated BEAS-2B cells (**Figure 6.3A** and **Figure 6.4A**). The percentage of monocytes expressing CD69 was not significantly altered when whole blood was incubated with conditioned media from BEAS-2B cells stimulated with TNF-α or uninfected cell lysate (**Figure 6.3A** and **Figure 6.4A**). Conditioned media from *C. pneumoniae* (IOL-207 isolate) stimulated BEAS-2B cells incubated with whole blood did not significantly alter the percentage of monocytes positive for CD69 expression, however, a significant increase in CD69 MFI was seen (**Figure 6.3B**).

Whole blood which had been incubated with conditioned media from BEAS-2B cells stimulated with the WA97001 *C. pneumoniae* isolate caused an increase in the percentage of monocytes expressing CD69 in comparison to uninfected cell lysate but only reached statistical significance a the highest concentration ($1x10^6$ ifu/ml; **Figure 6.4A**). Monocyte CD69 MFI was significantly increased after incubation with conditioned media from

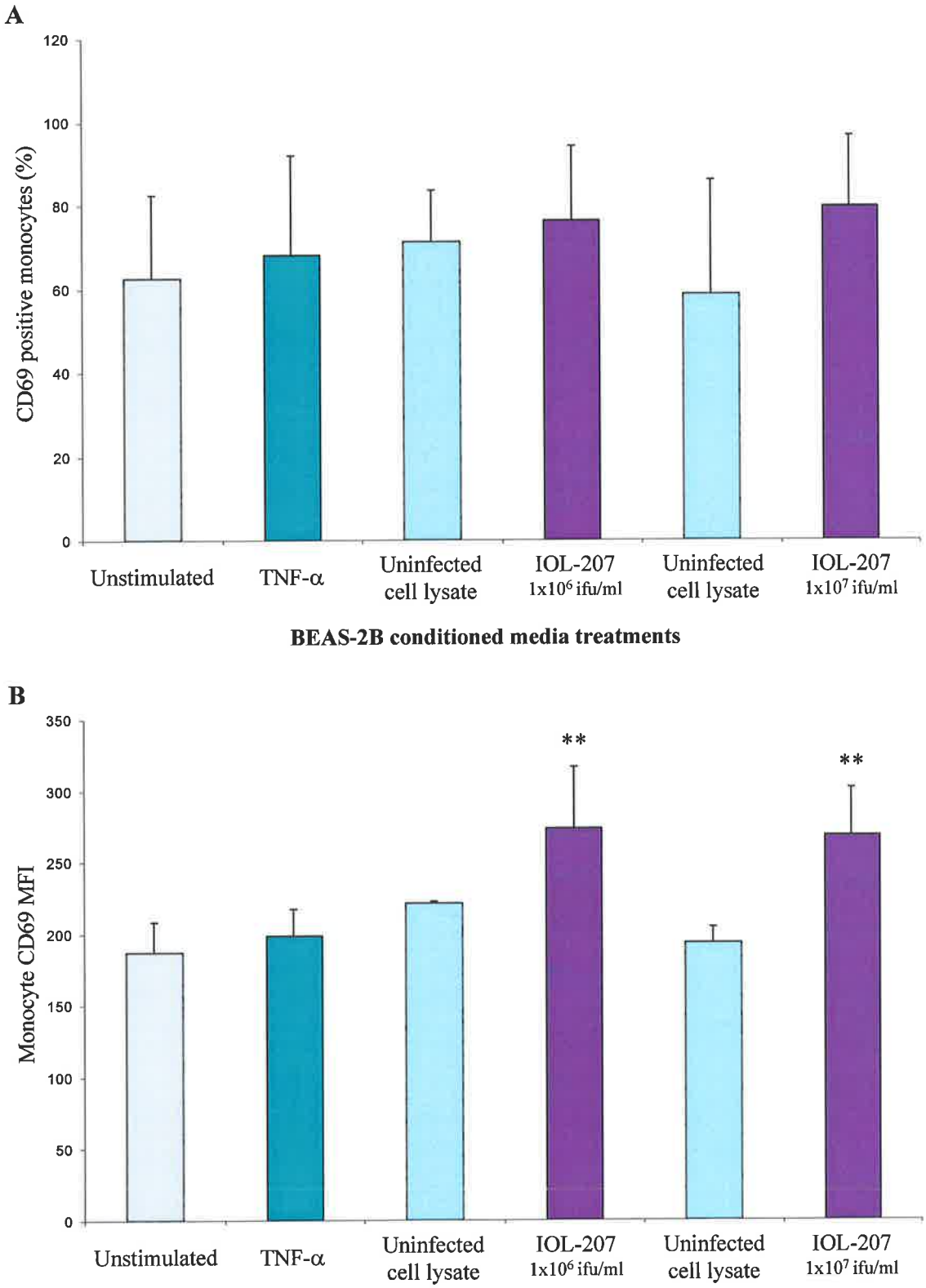
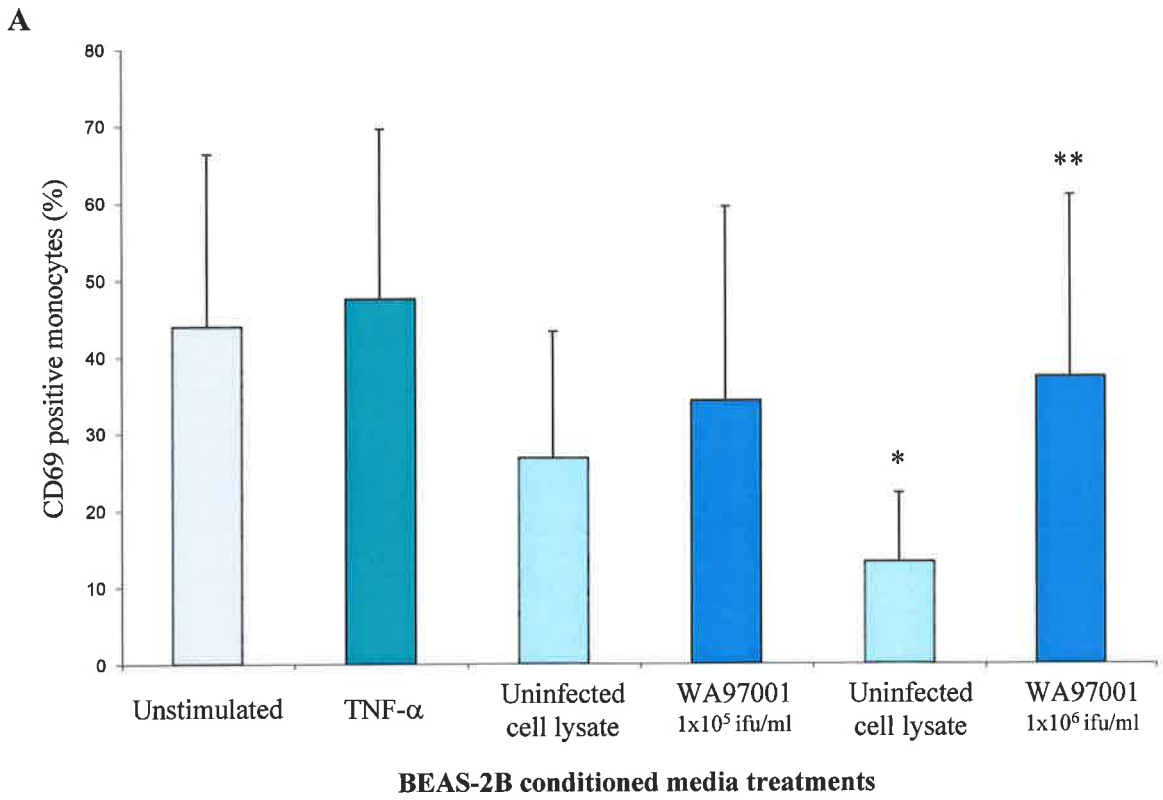


Figure 6.3: Effect of conditioned media from *C. pneumoniae* stimulated BEAS-2B cells on monocyte CD69 expression.

BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and monocyte CD69 expression detected using a CD69 PE monoclonal antibody and measured by flow cytometry. Graphs represent **A**) the percentage of monocytes expressing CD69 or **B**) monocyte CD69 MFI, after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* (IOL-207). Bars represent the mean ± SEM of 2 separate experiments performed in triplicate on whole blood obtained from 2 different volunteers. ** $p<0.05$ compared to comparable amount of uninfected cell lysate, mixed model ANOVA.



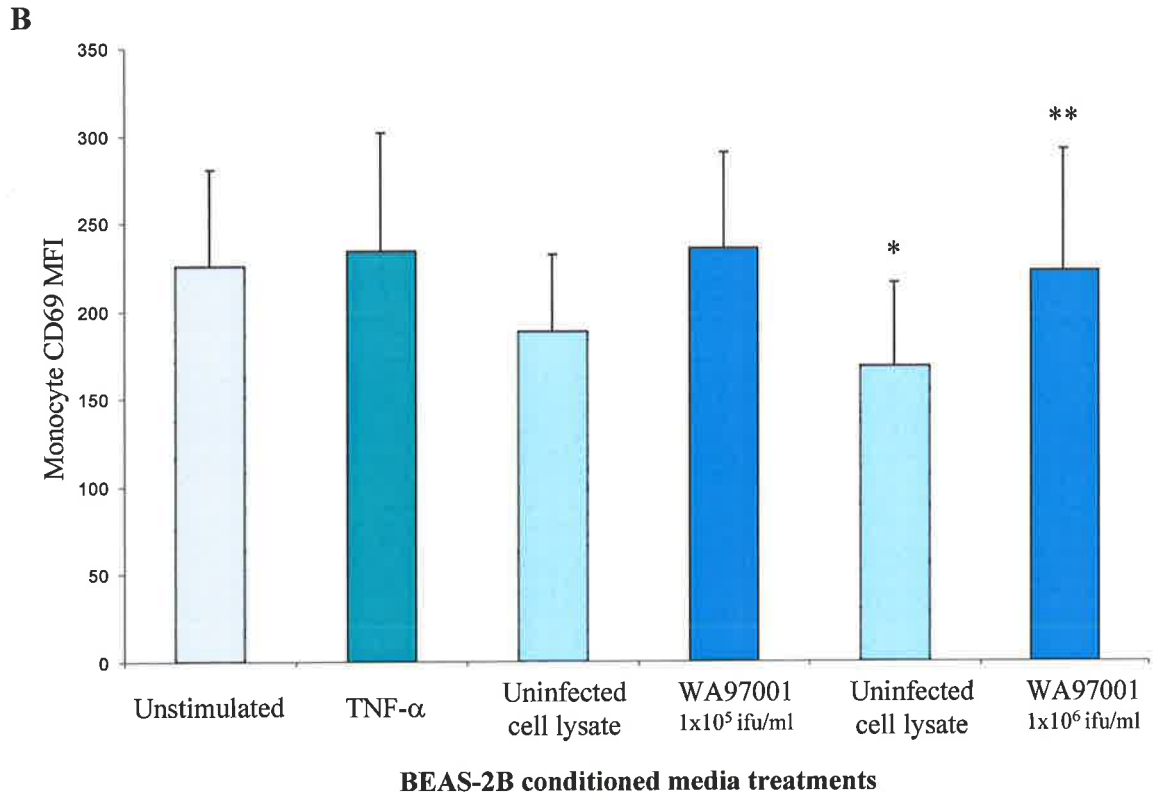


Figure 6.4: Conditioned media from *C. pneumoniae* (WA97001) stimulated BEAS-2B cells increases the percentage of monocytes expressing CD69.
BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and monocyte CD69 was detected using a CD69 PE monoclonal antibody and measured by flow cytometry. Graphs represent **A)** the percentage of monocytes expressing CD69 or **B)** monocyte CD69 MFI, after incubation with conditioned media from *C. pneumoniae* (WA97001) infected BEAS-2B cells. Bars represent the mean ± SEM of 3 separate experiments performed in triplicate on whole blood obtained from 3 different volunteers. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to uninfected cell lysate, mixed ANOVA.

BEAS-2B cells stimulated with WA97001, compared to those stimulated with uninfected cell lysate (**Figure 6.4B**). The reasons for this are unclear. However, this experiment may have been confounded by the fact that medium from uninfected lysate-incubated BEAS-2B cells actually had a negative effect on CD69 expression. Medium from the *C. pneumoniae* infected cells did not lead to an increase in CD69 compared to medium from unstimulated BEAS-2B cells.

Media obtained from wells incubated with the desired stimulus in the absence of BEAS-2B cells had minimal effects on the percentage of monocytes expressing CD69 and was expressed on less than 4% of monocytes under all conditions. These data suggest that the changes observed when whole blood was incubated with conditioned media from BEAS-2B cells is unlikely to be due to residual chlamydial organisms or proteins that may have attached to cell culture plates within the first 24 hour incubation period and became subsequently detached in the second time period.

To determine whether the changes in monocyte CD69 expression could be attributed to *C. pneumoniae*-induced IL-8 production, we stimulated whole blood with IL-8. The percentage of monocytes expressing CD69 was unchanged by incubation with IL-8 compared to unstimulated cells (**Table 6.3**).

Percentage of monocytes expressing CD69			
Subject	**Unstimulated**	***E. coli* LPS**	**IL-8**
1	2.84	1.48	3.18
2	6.05	0.91	1.76
3	2.15	5.92	1.15
Mean	3.68	2.77	2.03
SEM	1.20	1.58	0.60

Table 6.3: Direct stimulation of whole blood with IL-8 does not stimulate monocyte CD69 expression. Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), or IL-8 (5ng/ml). Monocytes were gated based on $CD14^+$ staining by flow cytometry and CD69 positive monocytes detected using an anti-human PE labelled monoclonal CD69 antibody. Data represents the mean percentage of monocytes expressing CD69 from 3 individual subjects, performed in triplicate.

Are neutrophils activated by conditioned media from C. pneumoniae infected airway epithelial cells?

After determining that monocytes were activated by conditioned media from *C. pneumoniae* infected airway epithelial cells, we addressed the same question with regard to neutrophils. To assess the activation status of neutrophils after stimulation with conditioned media from *C. pneumoniae* infected BEAS-2B cells we measured neutrophil expression of CD25 and CD69 by flow cytometry. Selection of neutrophils was based on forward and side scatter characteristics by flow cytometry as described in Chapter 2 (**Figure 2.15**). To assess the purity of this population we also stained neutrophils for CD16, which distinguishes eosinophils and neutrophils (192,363,370). The population of cells gated based on forward and side scatter characteristics was consistently over 95% pure as determined by the percentage of cells positive for CD16.

Consistent with observations in the previous chapter, CD25 was not expressed on neutrophils under any of the conditions tested. Similar to direct stimulation of whole blood with the IOL-207 *C. pneumoniae* isolate, the WA97001 isolate did not induce neutrophil CD25 expression.

We next assessed the activation status of neutrophils by measuring CD69 expression in response to conditioned media from *C. pneumoniae* stimulated epithelial cells. Between 60 and 80% of neutrophils expressed CD69 after incubation with media from unstimulated BEAS-2B cells. This suggests that a large proportion of neutrophils were activated. The percentage of neutrophils expressing CD69 was not affected by any of the treatments added to BEAS-2B cells, including the IOL-207 *C. pneumoniae* isolate (**Table 6.4A**). Similarly, analysis of neutrophil CD69 MFI remained unchanged after incubation of whole blood with conditioned media from BEAS-2B cells stimulated with the IOL-207 *C. pneumoniae* isolate (**Table 6.4B**).

Percentage of neutrophils expressing CD69						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	77.26	83.98	83.98	88.93	88.95	96.25
2	47.61	66.52	62.68	62.84	51.51	67.46
Mean	62.43	75.25	73.33	75.88	70.23	81.86
SEM	14.83	8.73	10.65	13.05	18.72	14.40

Table 6.4A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect the percentage of neutrophils expressing CD69.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD69 detected using an anti-human PE labelled monoclonal CD69 antibody. Data represents the mean percentage of neutrophils expressing CD69 from 2 individual subjects, performed in triplicate.

MFI of neutrophils expressing CD69						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	43.12	52.21	49.92	65.54	76.37	134.49
2	145.59	97.74	89.09	74.19	121.56	127.34
Mean	94.36	74.98	69.51	69.87	98.96	130.92
SEM	51.24	22.76	19.59	4.32	22.59	3.58

Table 6.4B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect neutrophil CD69 MFI.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD69 MFI was determined using an anti-human PE labelled monoclonal CD69 antibody. Data represents the mean MFI of neutrophils expressing CD69 from 2 individual subjects, performed in triplicate.

When whole blood was incubated with conditioned media obtained from BEAS-2B cells infected with the WA97001 *C. pneumoniae* isolate ($1x10^6$ ifu/ml), the percentage of neutrophils expressing CD69 was significantly reduced (**Figure 6.5**). Analysis of neutrophil CD69 MFI did not show any statistically significant changes under any of the conditions tested (**Table 6.5**).

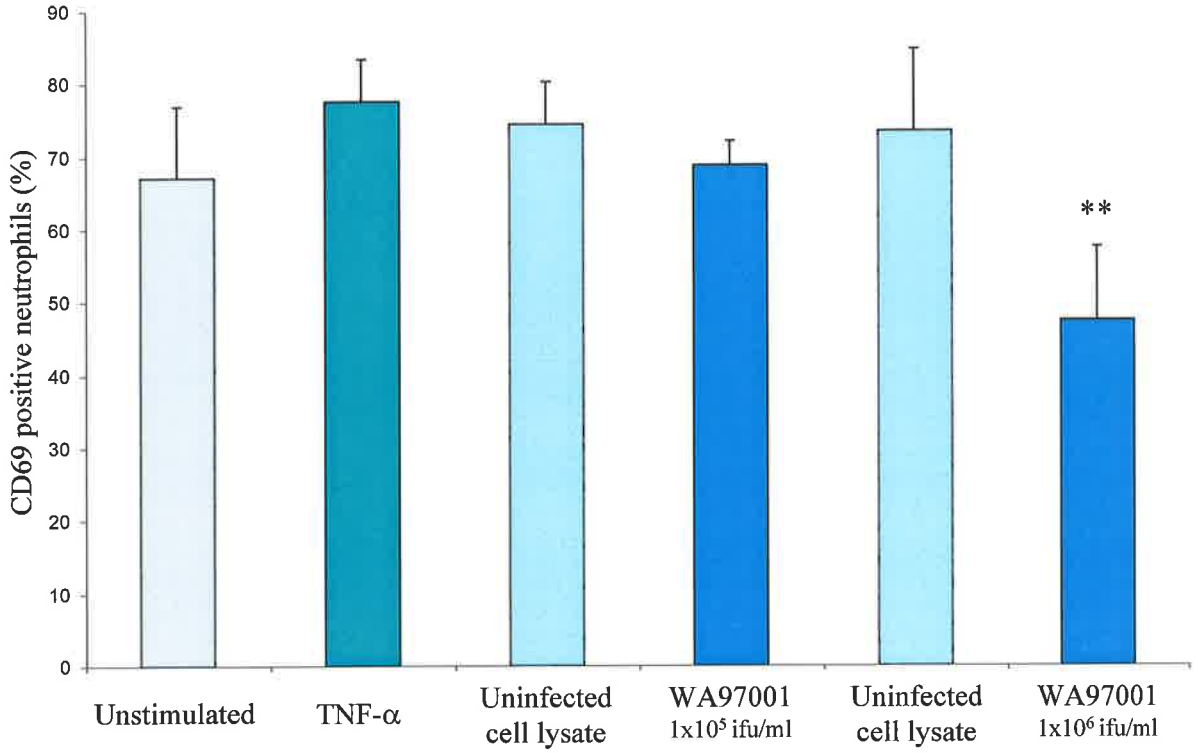


Figure 6.5: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells decreases the percentage of neutrophils expressing CD69.
BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and neutrophil CD69 expression detected using a CD69 PE monoclonal antibody and measured by flow cytometry. Bars represent the mean ± SEM of 3 separate experiments performed in triplicate on whole blood obtained from 3 different volunteers. ** $p<0.05$ compared to comparable amount of uninfected cell lysate, mixed ANOVA.

Mean fluorescent intensity of neutrophils expressing CD69						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	50.37	60.16	52.52	97.29	65.71	106.18
2	108.77	123.66	133.70	108.68	142.03	125.22
3	145.59	97.74	89.09	124.02	121.56	123.99
Mean	101.58	93.85	91.77	110.00	109.77	118.46
SEM	27.72	18.43	23.47	7.74	22.80	6.15

Table 6.5: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect the MFI of neutrophils expressing CD69.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD69 MFI was determined using an anti-human PE labelled monoclonal CD69 antibody. Data represents the mean MFI of neutrophils expressing CD69 from 3 individual subjects, performed in triplicate.

When whole blood was incubated with media obtained from wells in the absence of BEAS-2B cells (WA97001 isolate) no change in percentage CD69 positive cells or CD69 MFI was seen.

We investigated whether IL-8 released from *C. pneumoniae* infected BEAS-2B cells played a role in neutrophil activation. Direct stimulation of whole blood with *E. coli* LPS significantly increased the percentage of neutrophils expressing CD69 compared to unstimulated cells (**Table 6.6**). Incubation of whole blood with IL-8 did not cause a significant change in CD69 expression.

Percentage of neutrophils expressing CD69			
Subject	**Unstimulated**	***E. coli* LPS**	**IL-8**
1	10.49	43.42	2.94
2	16.88	73.30	21.42
3	42.63	39.55	4.06
Mean	23.33	52.09*	9.47
SEM	9.82	10.66	5.98

Table 6.6: Direct stimulation of whole blood with IL-8 does not stimulate neutrophil CD69 expression.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), or IL-8 (5ng/ml). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD69 positive neutrophils were detected using an anti-human PE labelled monoclonal CD69 antibody. Data represents the mean percentage of neutrophils expressing CD69 from 3 individual subjects, performed in triplicate. * $p<0.05$ compared to unstimulated cells, mixed ANOVA.

The expression of cell surface molecules such as CD25 and CD69 on leucocytes provides an indication as to whether the cells are in an activated state. In the previous chapter, we presented data that supports a direct role for *C. pneumoniae* activation of leucocytes. In the current set of experiments we have extended this data by showing that airway epithelial cells infected with *C. pneumoniae*, may also contribute to the activation status of monocytes and neutrophils. The expression of CD25 and CD69 on monocytes and neutrophils after exposure to conditioned media collected from *C. pneumoniae* infected BEAS-2B cells is summarised in **Table 6.7**.

Summary of activation marker expression on leucocytes after exposure to conditioned media from *C. pneumoniae* infected BEAS-2B cells				
Surface molecule	**Monocyte**		**Neutrophil**	
	IOL-207	**WA97001**	**IOL-207**	**WA97001**
CD25	↑	↔	nd	nd
CD69	↑	↑	↔	↓

Table 6.7: Conditioned media from *C. pneumoniae* infected airway epithelial cells modulates the expression of activation molecules on leucocytes.
nd, not detected; ↑, increased; ↓, decreased; ↔, unchanged.

Do C. pneumoniae infected airway epithelial cells modulate monocyte adhesion molecule expression?

After establishing that conditioned media from *C. pneumoniae* infected BEAS-2B cells alters the activation status of leucocytes, we next investigated whether the expression of adhesion molecules (CD11b and CD62L) are also modulated under these conditions. The expression of CD11b was detected on the entire population of monocytes and was unchanged by any of the conditions tested (**Table 6.8A** and **Table 6.8B**).

Percentage of monocytes expressing CD11b						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	99.86	99.89	99.95	99.98	99.80	99.81
2	99.98	100.00	99.98	99.96	99.97	99.87
Mean	99.92	99.94	99.97	99.97	99.89	99.84
SEM	0.06	0.06	0.01	0.01	0.09	0.03

Table 6.8A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not affect the percentage of monocytes expressing CD11b.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Monocytes were gated based on CD14 staining by flow cytometry and CD11b detected using an anti-human PE labelled monoclonal CD11b antibody. Data represents the mean percentage of monocytes expressing CD11b from 2 individual subjects, performed in triplicate.

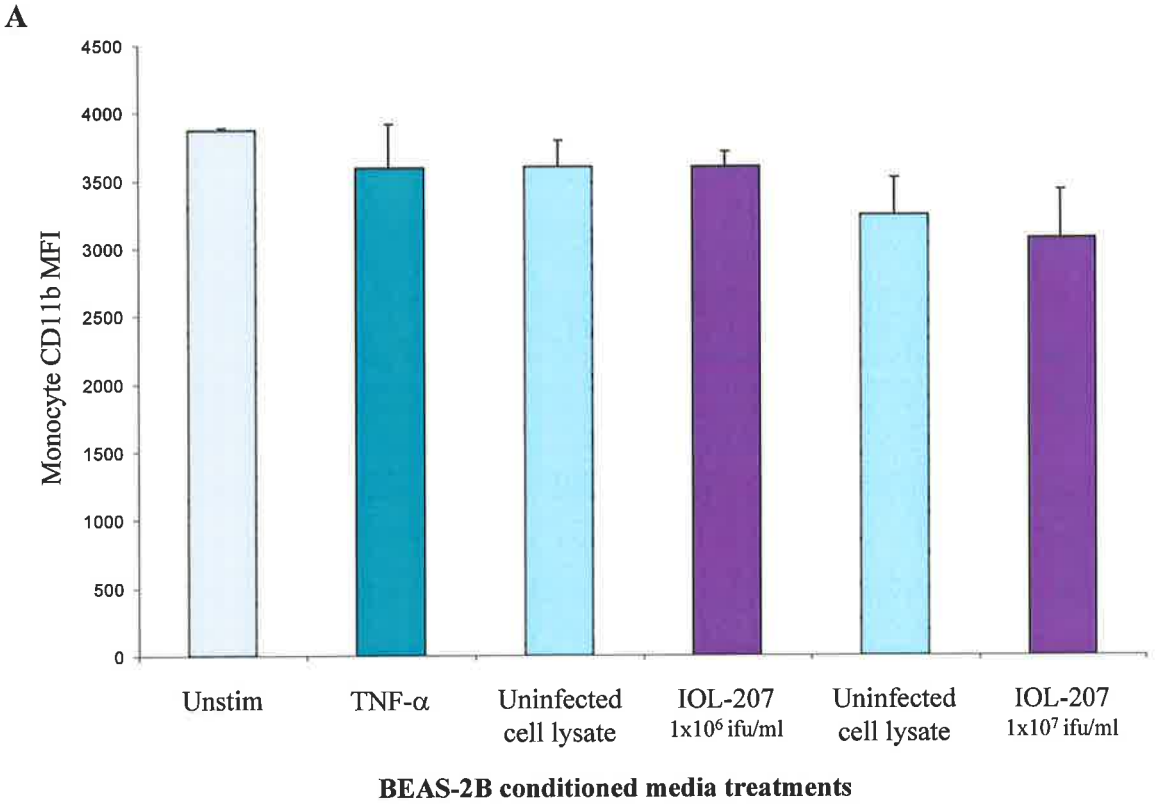
Percentage of monocytes expressing CD11b						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	99.86	99.89	99.95	99.23	99.80	99.40
2	97.86	98.44	98.22	98.30	99.02	98.40
3	99.74	99.46	99.45	92.67	99.26	93.36
Mean	99.15	99.26	99.21	96.73	99.36	97.05
SEM	0.65	0.43	0.51	2.05	0.23	1.87

Table 6.8B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not affect the percentage of monocytes expressing CD11b.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Monocytes were gated based on CD14 staining by flow cytometry and CD11b detected using an anti-human PE labelled monoclonal CD11b antibody. Data represents the mean percentage of monocytes expressing CD11b from 3 individual subjects, performed in triplicate.

When the MFI data was analysed, we detected a decrease in monocyte CD11b expression after incubation of whole blood with conditioned media from BEAS-2B cells infected with

C. pneumoniae (**Figure 6.6**). However, the decrease in monocyte CD11b MFI was only significantly decreased by the media from BEAS-2B cells infected with the WA97001 *C. pneumoniae* isolate.

Incubation of whole blood with media collected from wells with the desired stimulus in the absence of BEAS-2B cells did not significantly alter the percentage of monocytes expressing CD11b. The whole monocyte population was positive for CD11b. However, monocyte CD11b MFI was modulated by media collected from wells in the absence of BEAS-2B cells. When whole blood was incubated with media alone, from wells in the absence of BEAS-2B cells, monocyte CD11b MFI was significantly less than that observed for monocytes incubated with conditioned media from unstimulated BEAS-2B cells (**Table 6.9A** and **Table 6.9B**). Similar observations were observed for uninfected cell lysate and *C. pneumoniae* treatments with both IOL-207 and WA97001 isolates. However, when media was collected from wells incubated with the WA97001 *C. pneumoniae* isolate in the absence of BEAS-2B cells and incubated with whole blood overnight, monocyte CD11b MFI was significantly increased in comparison to the corresponding uninfected cell lysate treatment (**Table 6.9B**). This is in contrast to incubation of whole blood with media from WA97001 infected BEAS-2B cells, which resulted in a significant decrease in CD11b MFI (**Table 6.9B** and **Figure 6.6**). This data suggests that there may be some carry over effect from the *C. pneumoniae* or uninfected cell lysate preparations influencing monocyte CD11b expression intensity. However, conditioned media from *C. pneumoniae* infected BEAS-2B cells, compared with uninfected cell lysate treated cells, has a different effect on monocyte CD11b expression. These effects override baseline or background expression of monocyte CD11b observed with media obtained from wells in the absence of epithelial cells. Therefore, *C. pneumoniae* infected BEAS-2B cells may secrete products which play an important role in the regulation of monocyte CD11b expression, and cause a decrease in the intensity of monocyte CD11b surface expression.



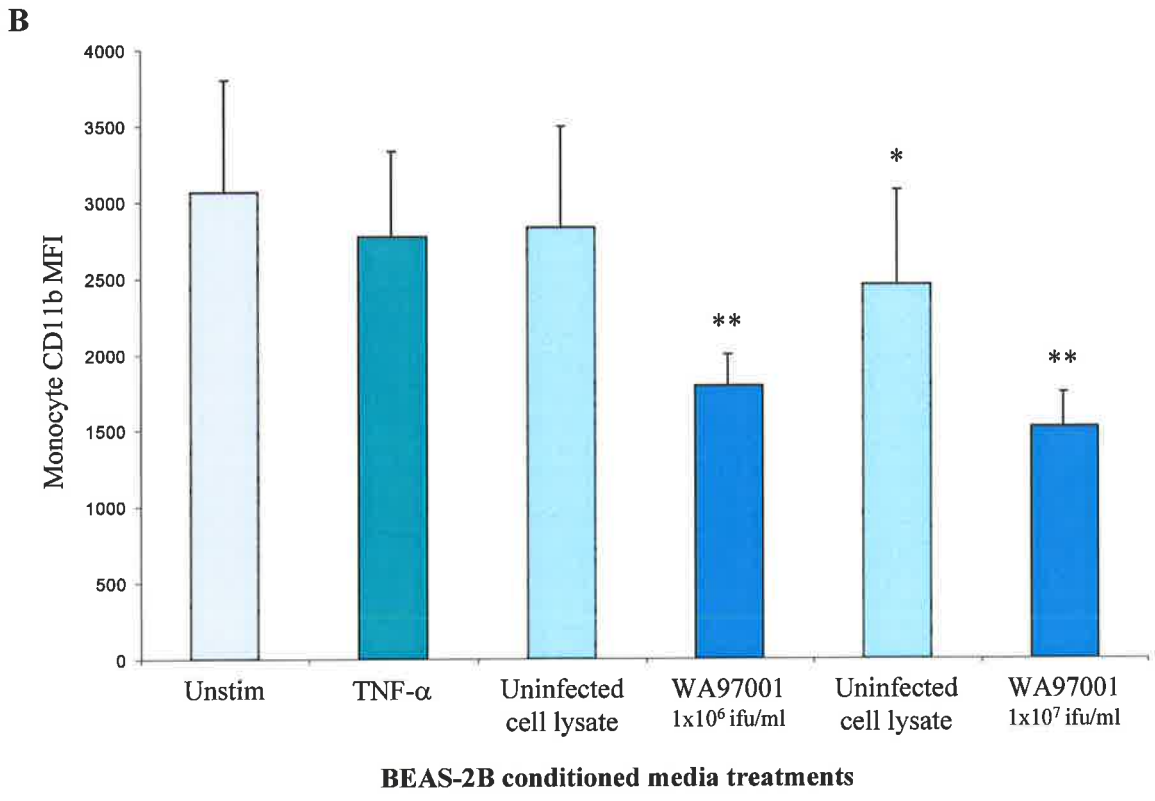


Figure 6.6: Conditioned media from *C. pneumoniae* infected BEAS-2B cells decreases monocyte CD11b MFI. BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and monocyte CD11b expression detected using a CD11b PE monoclonal antibody and measured by flow cytometry. Graphs represent MFI of monocytes expressing CD11b after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* **A)** IOL-207 or **B)** WA97001 isolates. Bars represent the mean ± SEM monocyte CD11b MFI of 2 (IOL-207) or 3 (WA97001) separate experiments performed in triplicate on whole blood obtained from 2 or 3 individual donors respectively. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of UCL.

Monocyte CD11b MFI											
	Conditioned media from BEAS-2B cells						Media from wells in the absence of BEAS-2B cells				
	Unstim	TNF-α	UCL	IOL $1x10^6$	UCL	IOL $1x10^7$	Unstim	UCL	IOL $1x10^6$	UCL	IOL $1x10^7$
1	3856.9	3256.9	3415.3	3706.1	2969.1	3423.5	2282.8	1952.8	2146.5	1220.7	1686.7
2	3885.8	3912.1	3783.6	3493.8	3517.5	2705.1	2181.4	1808.2	2504.4	na	na
Mean	3871.3	3584.5	3599.4	3599.9	3243.3	3064.3	2232.1	1880.5	2325.4	1220.7	1686.7
SEM	14.4	327.5	184.1	106.1	274.1	359.2	50.7	72.2	178.9	60.1	185.2
							*	**	† ‡	**	† ‡

Table 6.9A: Monocyte CD11b MFI after incubation with conditioned media obtained from wells in the presence or absence of BEAS-2B cells.
Whole blood was incubated overnight with media from wells treated with desired stimulus in the presence or absence of BEAS-2B cells before detecting CD11b monocyte expression by flow cytometry. Monocytes were detected using CD14 staining and side scatter characteristics and CD11b MFI was assessed by using a CD11b PE labelled monoclonal antibody by flow cytometry. Unstim = unstimulated; UCL = uninfected cell lysate; IOL = IOL-207 *C. pneumoniae* isolate; na = not assessed. * $p<0.05$ compared to Unstimulated BEAS-2B conditioned media. ** $p<0.05$ compared to corresponding UCL BEAS-2B conditioned media. † $p<0.05$ compared to corresponding WA BEAS-2B conditioned media. ‡ $p<0.05$ compared to media from wells incubated with a comparable amount of UCL in the absence of BEAS-2B cells.

Monocyte CD11b MFI											
	Conditioned media from BEAS-2B cells						Media from wells in the absence of BEAS-2B cells				
	Unstim	TNF-α	UCL	WA $1x10^5$	UCL	WA $1x10^6$	Unstim	UCL	WA $1x10^5$	UCL	WA $1x10^6$
1	3856.9	3256.9	3415.3	2078.3	2969.1	1730.3	2282.8	1952.8	2126.3	1220.7	2511.0
2	1601.1	1661.3	1510.8	1383.3	1216.7	1070.5	1921.5	1784.7	1633.7	1742.8	1760.5
3	3738.1	3396.9	3561.7	1908.7	3168.8	1755.6	2614.0	2385.9	3071.7	2308.6	3020.6
Mean	3065.4	2771.7	2829.3	1790.1	2451.5	1518.8	2272.8	2041.1	2277.2	1757.4	2430.7
SEM	732.9	556.6	660.6	209.2	620.1	224.2	199.9	179.0	421.9	314.1	365.9
							*	**	† ‡	**	† ‡

Table 6.9B: Monocyte CD11b MFI after incubation with conditioned media obtained from wells in the presence or absence of BEAS-2B cells.
Whole blood was incubated overnight with media from wells treated with desired stimulus in the presence or absence of BEAS-2B cells before detecting CD11b monocyte expression by flow cytometry. Monocytes were detected using CD14 staining and side scatter characteristics and CD11b MFI was assessed by using a CD11b PE labelled monoclonal antibody by flow cytometry. Unstim = unstimulated; UCL = uninfected cell lysate; WA = WA97001 *C. pneumoniae* isolate; * $p<0.05$ compared to Unstimulated BEAS-2B conditioned media. ** $p<0.05$ compared to corresponding UCL BEAS-2B conditioned media. † $p<0.05$ compared to corresponding WA BEAS-2B conditioned media. ‡ $p<0.05$ compared to media from wells incubated with a comparable amount of UCL in the absence of BEAS-2B cells.

As we have not previously investigated the direct effect of *C. pneumoniae* stimulation of whole blood with respect to CD11b expression we stimulated whole blood overnight and assessed monocyte CD11b MFI. In comparison to unstimulated monocytes, *E. coli* LPS

significantly reduced monocyte CD11b MFI (**Figure 6.7** and **Figure 6.8**). The highest amount of uninfected cell lysate also caused a significant reduction in monocyte CD11b MFI in comparison to unstimulated cells. Direct stimulation of whole blood with *C. pneumoniae* caused a further reduction of CD11b MFI which was significantly less than that observed after stimulation with uninfected cell lysate. We investigated whether these reductions could be reproduced by IL-8 stimulation of whole blood but monocyte CD11b MFI remained at a level similar to that seen with unstimulated cells. Thus, on balance we believe that the more significant effect of viable *C. pneumoniae* is a decrease in monocyte CD11b, which may occur either directly or via an interaction with airway epithelial cells. The studies using medium incubated at 37 degrees without BEAS-2B cells may have been confounded by degradation of residual material in the uninfected cell lysate or *C. pneumoniae* preparations under these circumstances.

The second adhesion molecule we investigated in this section of work was CD62L. We did not detect CD62L on the surface of whole blood monocytes incubated with conditioned media from unstimulated BEAS-2B cells (**Table 6.10**). In addition, CD62L was not detected on monocytes incubated with conditioned media from BEAS-2B cells under any of the conditions tested, including media from *C. pneumoniae* infected cells.

Percentage of monocytes expressing CD62L						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	0.33	0.04	0.02	0.03	0.04	0.00
2	0.16	0.17	0.28	0.39	0.28	0.40
Mean	0.24	0.11	0.15	0.21	0.16	0.20
SEM	0.08	0.07	0.13	0.18	0.12	0.20

Table 6.10A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not induce CD62L expression on monocytes.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Monocytes were gated based on CD14 staining by flow cytometry and CD62L detected using an anti-human FITC labelled monoclonal CD62L antibody. Data represents the mean percentage of monocytes expressing CD62L from 2 individual subjects, performed in triplicate.

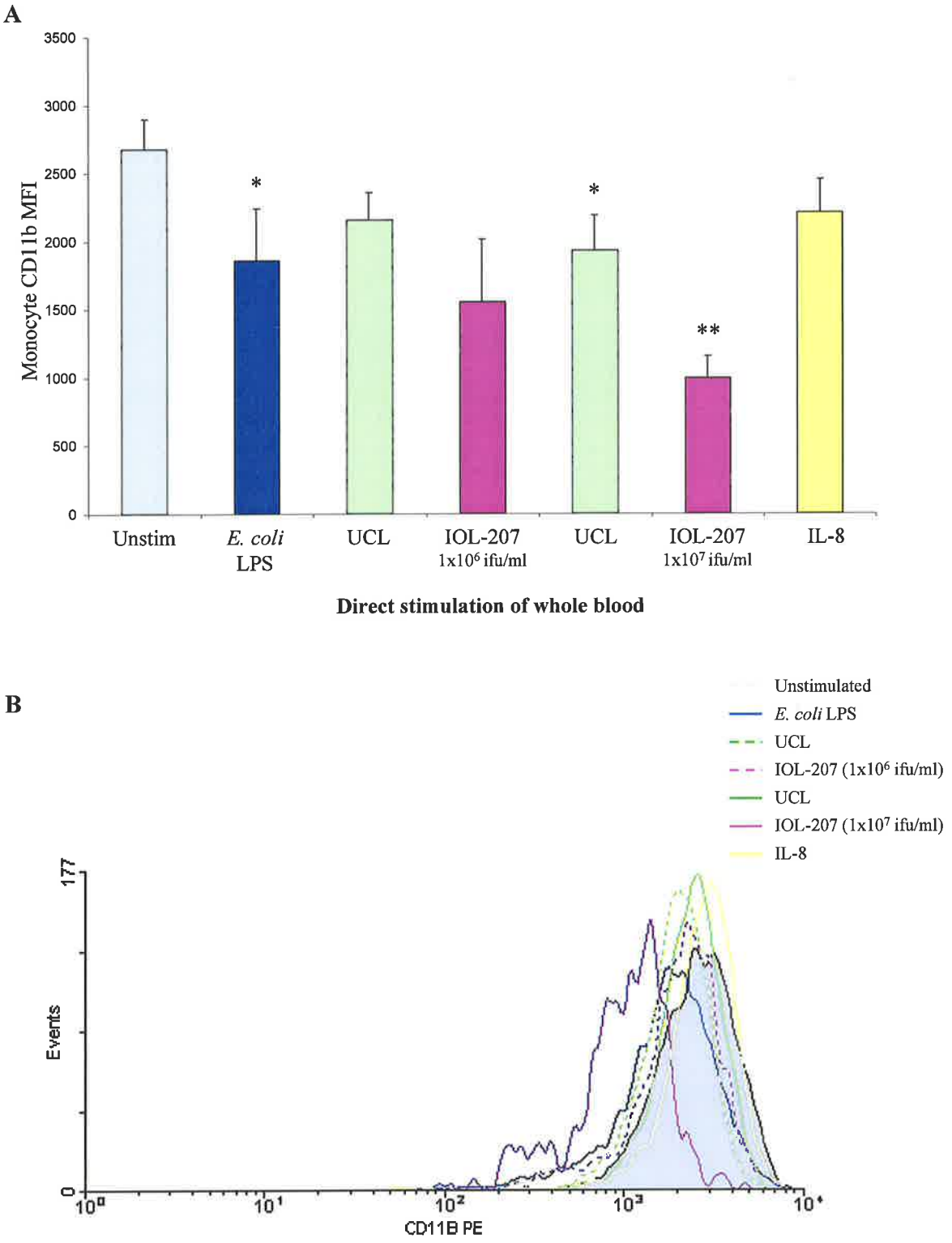


Figure 6.7: Direct stimulation of whole blood with *C. pneumoniae* decreases monocyte CD11b MFI.
Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), *C. pneumoniae* (IOL-207), uninfected cell lysate or IL-8 (5ng/ml). Monocytes were selected based on CD14 staining and side scatter characteristics by flow cytometry. Monocyte CD11b MFI was determined using a CD11b PE monoclonal antibody and measured by flow cytometry. **A)** Bars represent the mean ± SEM of 2 separate experiments performed in duplicate. **B)** Histogram is representative of monocytes displaying reduced CD11b MFI with *C. pneumoniae* stimulation (IOL-207) with respect to relevant control. * $p<0.05$ compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to comparable amount of UCL, mixed ANOVA.

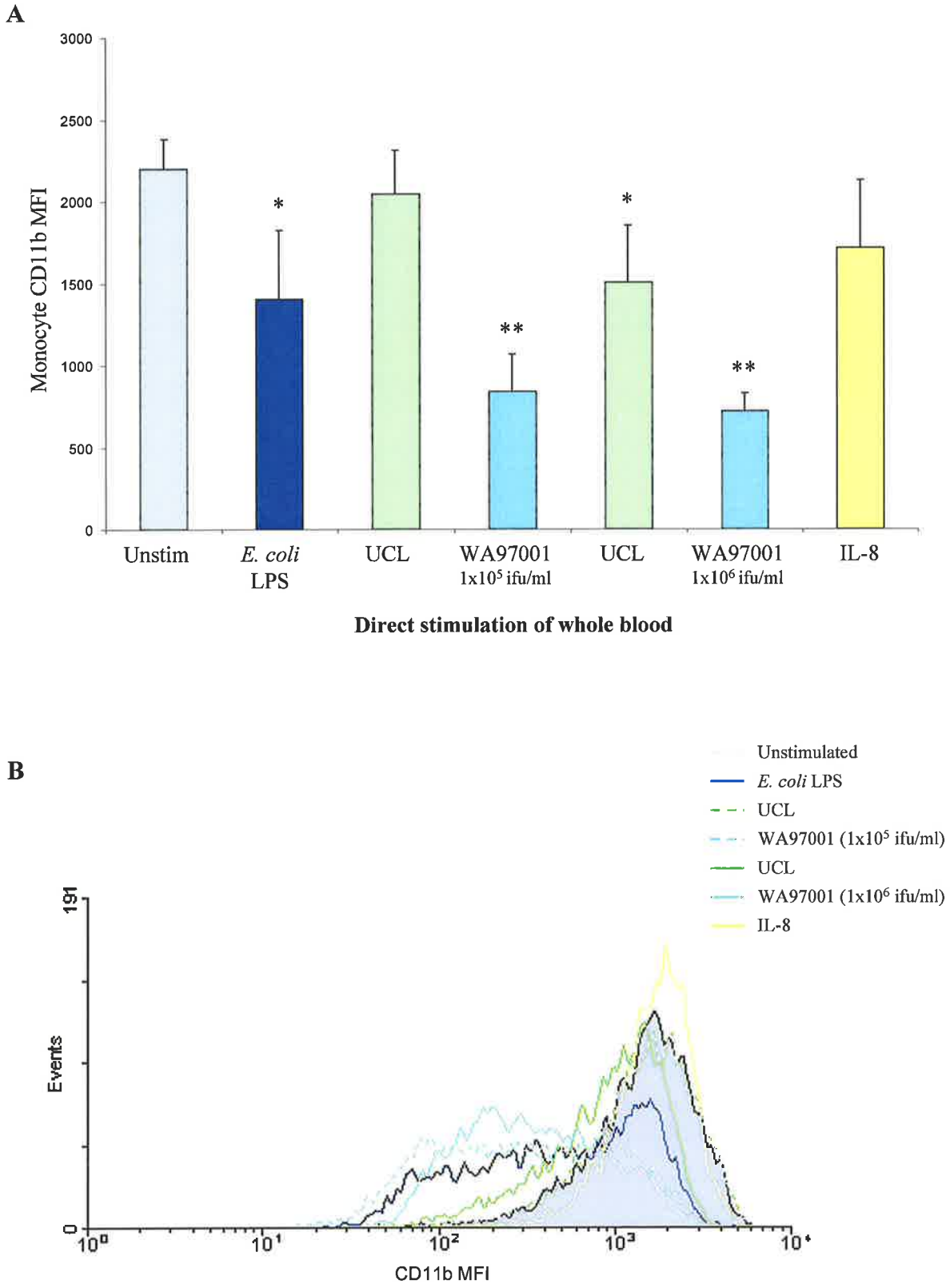


Figure 6.8: Direct stimulation of whole blood with *C. pneumoniae* decreases monocyte CD11b MFI. Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), *C. pneumoniae* (WA97001), uninfected cell lysate or IL-8 (5ng/ml). Monocytes were selected based on CD14 staining and side scatter characteristics by flow cytometry. Monocyte CD11b MFI was determined using a CD11b PE monoclonal antibody and measured by flow cytometry. **A**) Bars represent the mean ± SEM of 3 separate experiments performed in duplicate. **B**) Histogram is representative of monocytes displaying reduced CD11b MFI with *C. pneumoniae* stimulation (WA97001) with respect to relevant control. * $p<0.05$ compared to unstimulated cells, mixed ANOVA. ** $p<0.05$ compared to comparable amount of UCL, mixed ANOVA.

Percentage of monocytes expressing CD62L						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	0.33	0.04	0.02	0.10	0.04	0.18
2	0.45	0.35	0.45	0.82	0.60	0.62
3	0.47	0.39	0.30	0.42	0.36	0.57
Mean	0.42	0.26	0.26	0.45	0.33	0.46
SEM	0.05	0.11	0.12	0.21	0.16	0.14

Table 6.10B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does induce CD62L expression on monocytes.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Monocytes were gated based on CD14 staining by flow cytometry and CD62L detected using an anti-human FITC labelled monoclonal CD62L antibody. Data represents the mean percentage of monocytes expressing CD62L from 3 individual subjects, performed in triplicate.

We therefore ascertained whether CD62L was detectable on resting whole blood monocytes or by direct stimulation with *E. coli* LPS or *C. pneumoniae*. We did not detect any monocytes expressing CD62L under these conditions.

Do C. pneumoniae infected airway epithelial cells modulate neutrophil adhesion molecule expression?

After determining that *C. pneumoniae* infected epithelial cells modulated adhesion molecules on whole blood monocytes, we addressed the same question with regard to neutrophils. We therefore assessed the expression of CD11b on neutrophils after exposure to conditioned media from *C. pneumoniae* infected BEAS-2B cells. The expression of CD11b was detected on the entire population of neutrophils and was unchanged by any of the conditions tested (**Table 6.11A** and **Table 6.11B**), including incubation of whole blood neutrophils with media from *C. pneumoniae* infected cells.

Percentage of neutrophils expressing CD11b						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	99.84	99.98	99.98	100.00	99.99	98.91
2	98.90	99.90	99.72	99.88	98.88	99.76
Mean	99.37	99.94	99.85	99.94	99.43	99.34
SEM	0.47	0.04	0.13	0.06	0.56	0.42

Table 6.11A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect the percentage of neutrophils expressing CD11b.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD11b detected using an anti-human PE labelled monoclonal CD11b antibody. Data represents the mean percentage of neutrophils expressing CD11b from 2 individual subjects, performed in triplicate.

Percentage of neutrophils expressing CD11b						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	99.97	99.95	99.97	99.96	99.98	99.87
2	99.95	99.91	99.93	99.91	99.72	99.76
3	98.90	99.90	99.72	99.40	98.88	98.29
Mean	99.60	99.92	99.87	99.76	99.53	99.31
SEM	0.35	0.02	0.08	0.18	0.33	0.51

Table 6.11B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not effect the percentage of neutrophils expressing CD11b.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD11b detected using an anti-human PE labelled monoclonal CD11b antibody. Data represents the mean percentage of neutrophils expressing CD11b from 3 individual subjects, performed in triplicate.

When the MFI data was analysed, there was no change in neutrophil CD11b MFI when comparing whole blood which was incubated with conditioned media from unstimulated BEAS-2B cells or from TNF-α stimulated BEAS-2B cells (**Figure 6.9** and **Figure 6.10**). However, we detected a significant increase in CD11b MFI on neutrophils which had been incubated with conditioned media from BEAS-2B cells which had been infected with either *C. pneumoniae* isolate, in comparison to whole blood neutrophils incubated with conditioned media from BEAS-2B cells incubated with uninfected cell lysate (**Figure 6.9** and **Figure 6.10**).

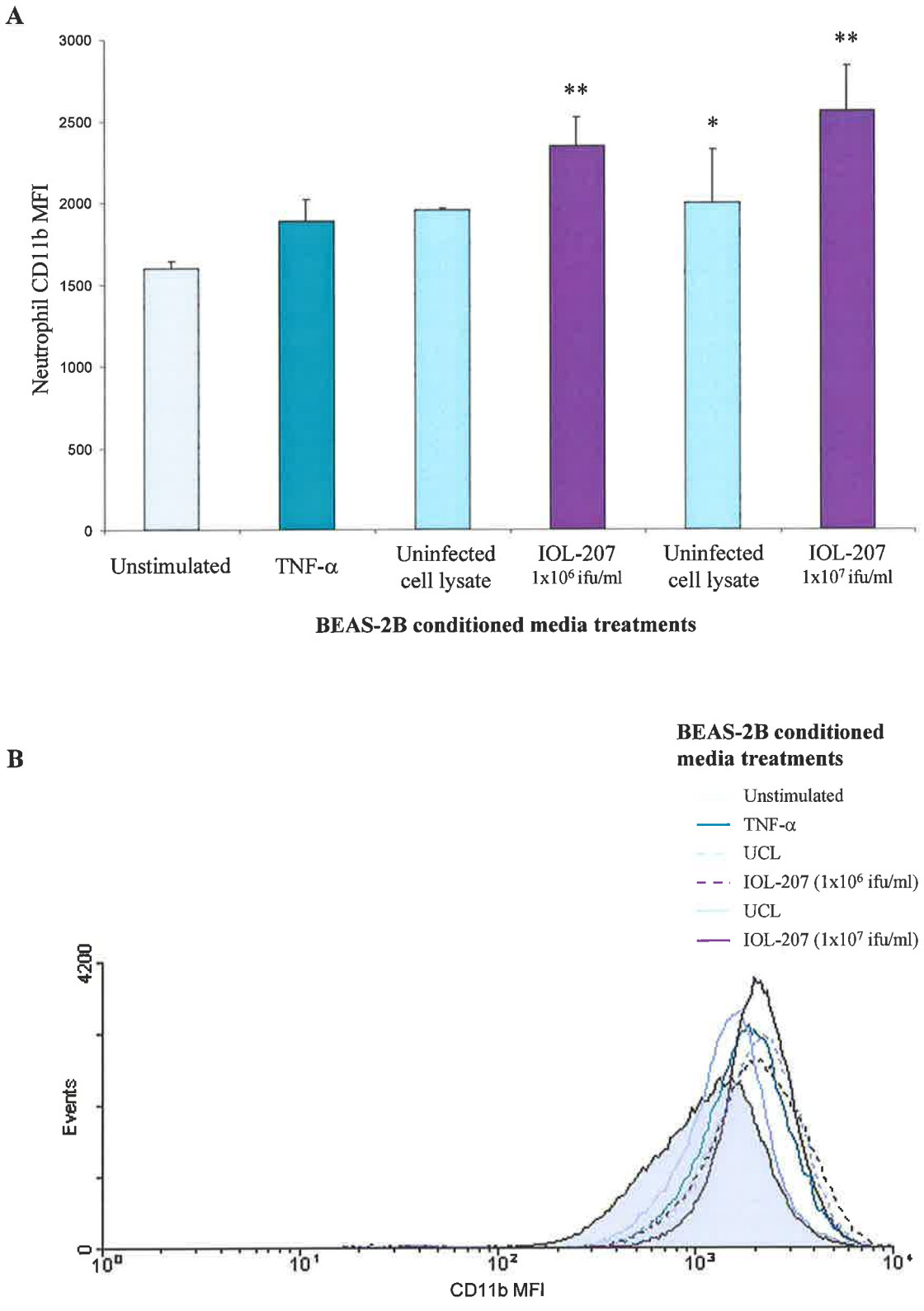


Figure 6.9: Conditioned media from *C. pneumoniae* (IOL-207) stimulated BEAS-2B cells increases neutrophil CD11b MFI.

BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and neutrophil CD11b MFI determined using a CD11b PE monoclonal antibody and measured by flow cytometry. **A)** Graph represents neutrophil CD11b MFI where bars represent the mean ± SEM of 2 separate experiments performed in triplicate on whole blood obtained from 2 different volunteers. **B)** Representative histogram of increased neutrophil CD11b MFI, after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* (IOL-207). * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of UCL, mixed ANOVA.

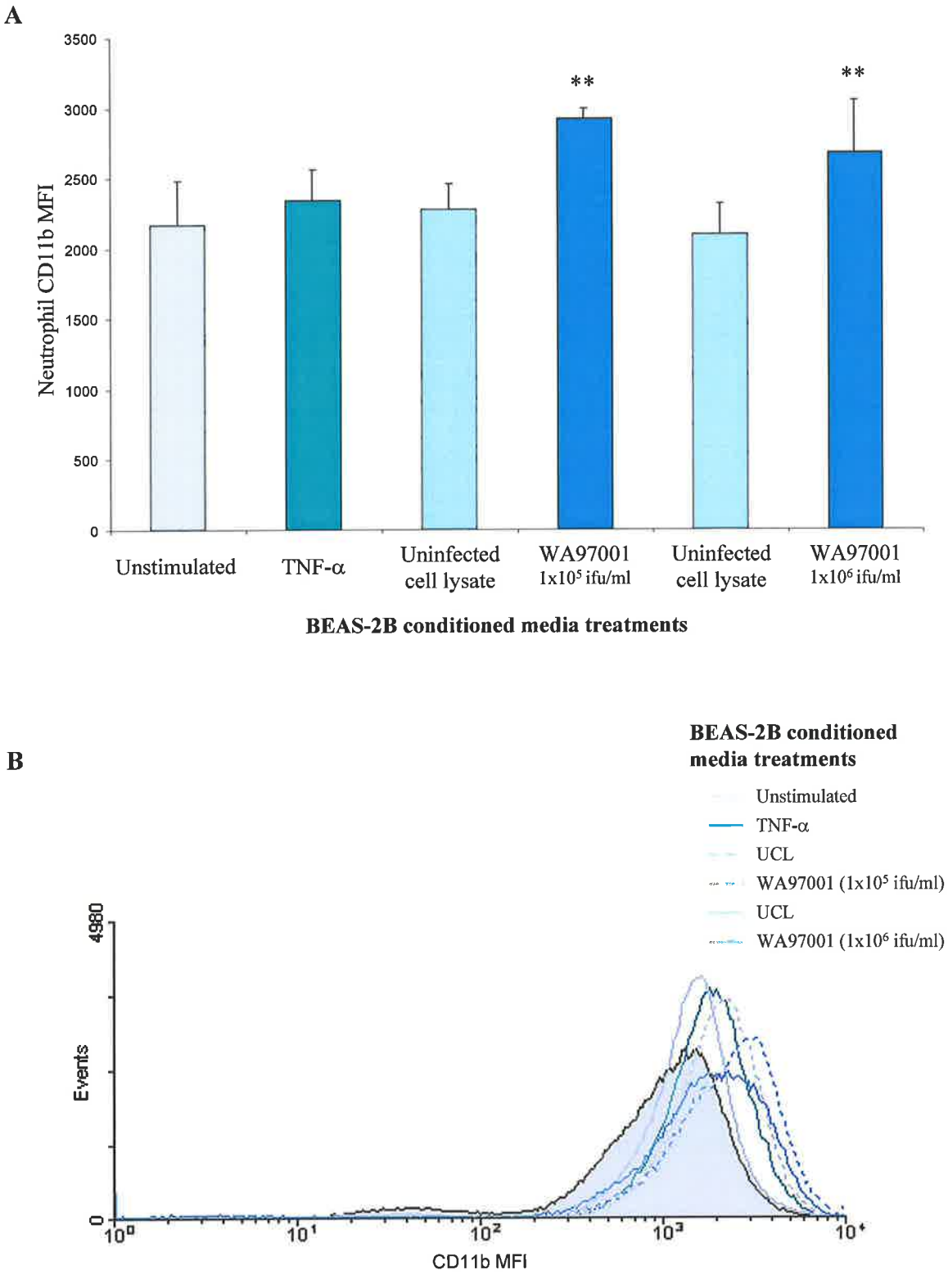


Figure 6.10: Conditioned media from *C. pneumoniae* (WA97001) stimulated BEAS-2B cells increases neutrophil CD11b MFI.

BEAS-2B cells were incubated overnight with *C. pneumoniae*, uninfected cell lysate, TNF-α (20ng/ml) or media alone. Cell monolayers were washed with PBS before replacing media and incubated for a further 24 hours. Conditioned media was added to whole blood overnight and neutrophil CD11b MFI determined using a CD11b PE monoclonal antibody and measured by flow cytometry. **A)** Graph represents neutrophil CD11b MFI where bars represent the mean ± SEM of 3 separate experiments performed in triplicate on whole blood obtained from 3 different volunteers. **B)** Representative histogram of increased neutrophil CD11b MFI, after incubation with conditioned media from BEAS-2B cells stimulated with *C. pneumoniae* (WA97001). ** $p<0.05$ compared to comparable amount of UCL, mixed ANOVA.

Similar to the data obtained when examining monocyte adhesion molecule expression, the percentage of neutrophils expressing CD11b was not significantly changed after incubation of whole blood with media collected from wells without BEAS-2B cells. All neutrophils were CD11b positive. However, when a comparison was made between whole blood incubated with media from unstimulated BEAS-2B cells, to media collected in the absence of BEAS-2B cells, neutrophil CD11b MFI was higher (**Table 6.12A** and **Table 6.12B**). Similarly, neutrophil CD11b MFI was higher after incubation with conditioned media from BEAS-2B cells incubated with uninfected cell lysate, in comparison to media collected from wells with uninfected cell lysate in the absence of BEAS-2B cells. When whole blood was incubated with conditioned media from *C. pneumoniae* stimulated BEAS-2B cells, neutrophil CD11b MFI was significantly higher than that detected on neutrophils incubated with media with *C. pneumoniae* in the absence of BEAS-2B cells. This data suggests that although neutrophils express a baseline intensity of CD11b, and it may be modulated by residual *C. pneumoniae*, conditioned media from *C. pneumoniae* infected BEAS-2B cells, modulates neutrophil CD11b expression, over and above that observed in the absence of infected cells. Therefore, it is feasible that *C. pneumoniae* infected epithelial cells secrete factors that may further modulate neutrophil CD11b expression. These products cause an increase in neutrophil CD11b expression.

Neutrophil CD11b MFI											
	Conditioned media from BEAS-2B cells						Media from wells in the absence of BEAS-2B cells				
	Unstim	TNF-α	UCL	IOL $1x10^5$	UCL	IOL $1x10^6$	Unstim	UCL	IOL $1x10^6$	UCL	IOL $1x10^7$
1	1641.0	1750.6	1960.8	2521.9	2318.5	2835.7	1279.5	1256.5	1627.0	931.5	1294.4
2	1555.1	2014.6	1939.8	2160.3	1670.7	2280.3	1243.4	1245.7	1415.8	na	na
Mean	1598.1	1882.6	1950.3	2341.1	1994.6	2558.0	1261.5	1251.1	1521.4	931.5	1294.4
SEM	42.9	131.9	10.5	180.8	323.9	277.6	18.0	5.4	105.5	68.17	151.76
								**	† ‡	**	† ‡

Table 6.12A: Neutrophil CD11b MFI after incubation with conditioned media obtained from wells in the presence or absence of BEAS-2B cells.
Whole blood was incubated overnight with media from wells treated with desired stimulus in the presence or absence of BEAS-2B cells before detecting CD11b neutrophil expression by flow cytometry. Neutrophils were detected using forward and side scatter characteristics and CD11b MFI was assessed by using a CD11b PE labelled monoclonal antibody by flow cytometry. Unstim = unstimulated; UCL = uninfected cell lysate; IOL = IOL-207 *C. pneumoniae* isolate; na = not assessed ** $p<0.05$ compared to corresponding UCL BEAS-2B conditioned media. † $p<0.05$ compared to corresponding IOL BEAS-2B conditioned media. ‡ $p<0.05$ compared to media from wells incubated with a comparable amount of UCL in the absence of BEAS-2B cells.

Neutrophil CD11b MFI											
	Conditioned media from BEAS-2B cells						Media from wells in the absence of BEAS-2B cells				
	Unstim	TNF-α	UCL	WA $1x10^5$	UCL	WA $1x10^6$	Unstim	UCL	WA $1x10^5$	UCL	WA $1x10^6$
1	2364.0	2244.8	2320.7	3069.7	2403.8	3329.6	1407.2	1182.6	1686.4	1240.5	1983.4
2	2587.3	2754.6	2562.3	2837.7	2215.7	2670.5	1498.7	1306.8	1770.2	1410.9	1843.9
3	1555.1	2014.6	1939.8	2844.0	1670.7	2029.5	1243.4	1245.7	1366.3	931.5	2484.2
Mean	2168.8	2338.0	2274.3	2917.1	2096.7	2676.5	1383.1	1245.0	1607.6	1194.3	2103.9
SEM	313.5	218.6	181.2	76.3	219.8	375.3	74.6	35.8	123.0	140.3	194.4
							*	**	† ‡	**	† ‡

Table 6.12B: Neutrophil CD11b MFI after incubation with conditioned media obtained from wells in the presence or absence of BEAS-2B cells.
Whole blood was incubated overnight with media from wells treated with desired stimulus in the presence or absence of BEAS-2B cells before detecting CD11b neutrophil expression by flow cytometry. Neutrophils were detected using forward and side scatter characteristics and CD11b MFI was assessed by using a CD11b PE labelled monoclonal antibody by flow cytometry. Unstim = unstimulated; UCL = uninfected cell lysate; WA = WA97001 *C. pneumoniae* isolate; na = not assessed * $p<0.05$ compared to Unstimulated BEAS-2B conditioned media. ** $p<0.05$ compared to corresponding UCL BEAS-2B conditioned media. † $p<0.05$ compared to corresponding IOL BEAS-2B conditioned media. ‡ $p<0.05$ compared to media from wells incubated with a comparable amount of UCL in the absence of BEAS-2B cells.

As with monocyte CD11b expression, we have not previously examined neutrophil CD11b expression in response to direct stimulation of whole blood with *C. pneumoniae*. We therefore, incubated whole blood overnight with *E. coli* LPS, *C. pneumoniae*, or an equivalent

amount of uninfected cell lysate and assessed neutrophil CD11b MFI. Incubation of whole blood with *E. coli* LPS significantly increased neutrophil CD11b MFI, compared to unstimulated cells. Direct stimulation of whole blood with either *C. pneumoniae* isolate caused an increase in neutrophil CD11b MFI but only reached statistical significance at the lower concentrations of *C. pneumoniae* (**Figure 6.11**). We have previously shown that *C. pneumoniae* infected BEAS-2B cells increase their secretion of IL-8 (see Chapter 3). We therefore stimulated whole blood with IL-8 and observed that neutrophil CD11b MFI was significantly reduced in comparison to unstimulated cells (**Figure 6.11**). Therefore it would appear that the *C. pneumoniae* – induced increase in CD11b is not mediated by IL-8.

We also assessed the expression of CD62L on neutrophils after incubation with conditioned media from BEAS-2B cells. We did not consistently detect CD62L on the surface of whole blood neutrophils incubated with conditioned media from unstimulated BEAS-2B cells (**Table 6.13**). In addition the percentage of neutrophils expressing CD62L remained at 2% or less under all other conditions tested.

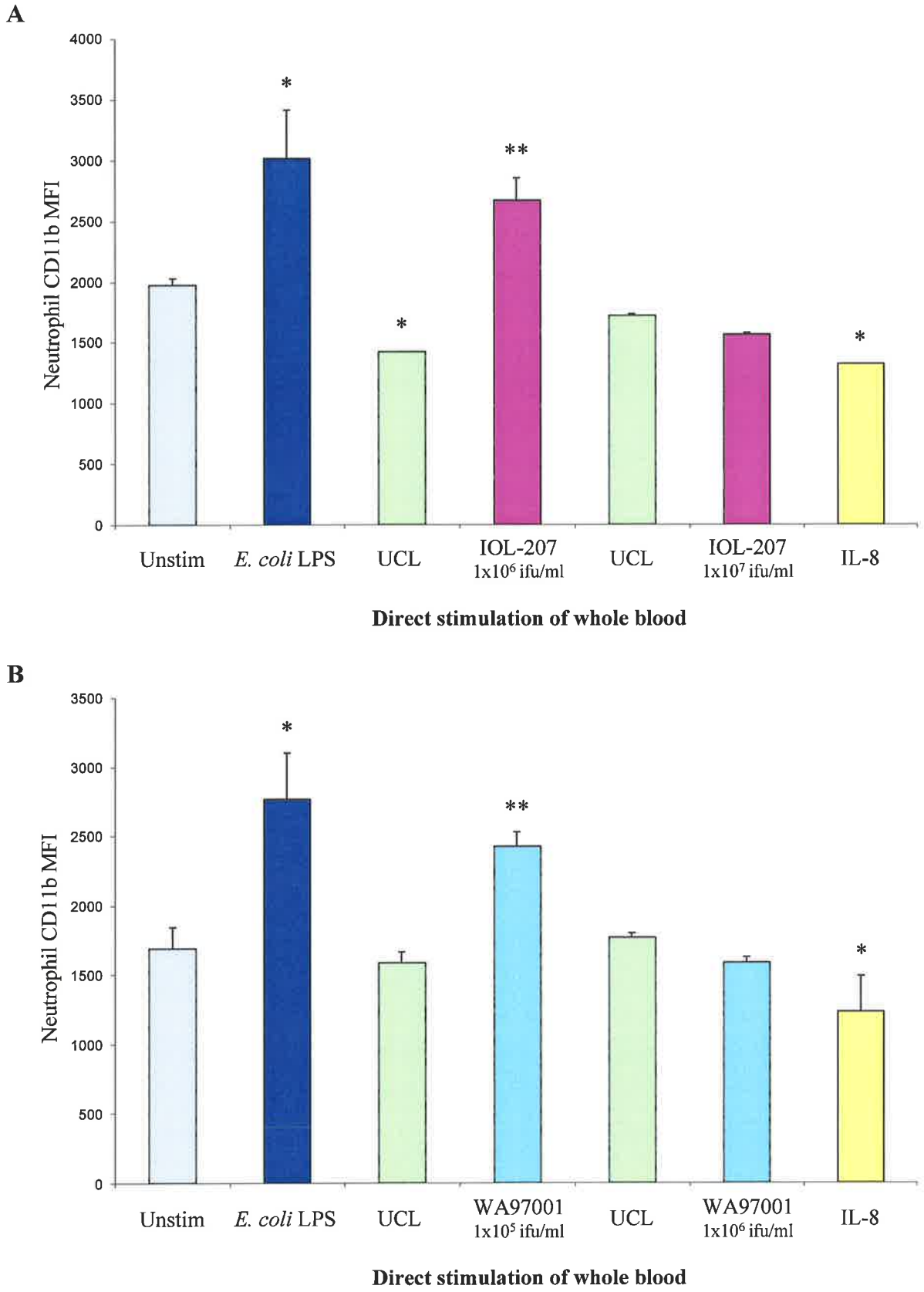


Figure 6.11: Direct stimulation of whole blood with *C. pneumoniae* increases neutrophil CD11b MFI. Whole blood was incubated overnight with *E. coli* LPS (100ng/ml), uninfected cell lysate (UCL), *C. pneumoniae* **A)** IOL-207 or **B)** WA97001, or IL-8 (5ng/ml). Neutrophils were gated based on FSC and SSC characteristics by flow cytometry and CD11b MFI was determined using an anti-human CD11b PE labelled monoclonal antibody. Bars represent the mean ± SEM of 2 (IOL-207) or 3 (WA97001) separate experiments performed in duplicate. * $p<0.05$ compared to unstimulated, mixed ANOVA. ** $p<0.05$ compared to comparable amount of UCL, mixed ANOVA.

Percentage of neutrophils expressing CD62L						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**IOL-207 $1x10^6$**	**UCL**	**IOL-207 $1x10^7$**
1	4.46	2.59	4.37	1.30	0.72	0.52
2	0.37	0.05	0.19	0.26	0.43	0.06
Mean	2.41	1.32	2.28	0.78	0.58	0.29
SEM	2.05	1.27	2.09	0.52	0.15	0.23

Table 6.13A: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does not induce CD62L expression on neutrophils.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (IOL-207). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD62L detected using an anti-human FITC labelled monoclonal CD62L antibody. Data represents the mean percentage of neutrophils expressing CD62L from 2 individual subjects, performed in triplicate.

Percentage of neutrophils expressing CD62L						
Subject	**Unstimulated**	**TNF-α (20ng/ml)**	**UCL**	**WA97001 $1x10^5$**	**UCL**	**WA97001 $1x10^6$**
1	4.24	2.98	2.59	0.39	1.77	0.35
2	0.32	0.30	0.30	0.55	0.48	0.39
3	0.37	0.05	0.19	0.81	0.43	0.13
Mean	1.64	1.11	1.02	0.58	0.89	0.29
SEM	1.30	0.94	0.78	0.12	0.44	0.08

Table 6.13B: Conditioned media from *C. pneumoniae* stimulated BEAS-2B cells does induce CD62L expression on neutrophils.
Whole blood was incubated overnight with conditioned media from BEAS-2B cells stimulated with TNF-α (20ng/ml), uninfected cell lysate or *C. pneumoniae* (WA97001). Neutrophils were gated based on forward and side scatter characteristics by flow cytometry and CD62L detected using an anti-human FITC labelled monoclonal CD62L antibody. Data represents the mean percentage of neutrophils expressing CD62L from 3 individual subjects, performed in triplicate.

Due to these results, we investigated CD62L expression on neutrophils from whole blood. As with the above mentioned studies, CD62L was only barely detectable on less than 2% of neutrophils in unstimulated blood, and was unchanged after direct stimulated with *E. coli* LPS or *C. pneumoniae*.

The expression of adhesion molecules on monocytes and neutrophils not only facilitate adhesion between cells but are also involved in other cellular processes such as phagocytosis. In the previous chapter we presented data that supports a role for *C. pneumoniae* in the

modulation of various leucocyte adhesion molecules. In the current chapter we have shown that airway epithelial cells infected with *C. pneumoniae* may also contribute to the regulation of leucocyte adhesion molecule expression. The modulation of monocyte and neutrophil expression of CD11b and CD62L by conditioned media from *C. pneumoniae* infected airway epithelial cells is summarised in **Table 6.14**.

Summary of adhesion molecule expression on leucocytes after exposure to conditioned media from *C. pneumoniae* infected BEAS-2B cells

Surface molecule	Monocyte		Neutrophil	
	IOL-207	WA97001	IOL-207	WA97001
CD11b	↔	↓	↑	↑
CD62L	nd	nd	nd	nd

Table 6.14: Conditioned media from *C. pneumoniae* infected airway epithelial cells modulates the expression of adhesion molecules on leucocytes in a cell specific manner.
nd, not detected; ↑, increased; ↓, decreased; ↔, unchanged.

Discussion

The airway epithelium is ideally located to protect the respiratory system from pollutants, noxious stimuli and potential pathogens. It is actively involved in the immune response to such stimuli and is a source of a range of cytokines and inflammatory mediators. In earlier chapters we have shown that two such mediators, IL-8 and IL-6, are released from the epithelium in response to infection with *C. pneumoniae*. These cytokines are involved in the chemotaxis and activation of leucocytes, including neutrophils and monocytes. We therefore investigated whether conditioned media from *C. pneumoniae* infected airway epithelial cells modulated the activation status of monocytes and neutrophils, as indicated by surface molecule expression. We found that conditioned media from *C. pneumoniae* infected BEAS-2B cells increased the expression of CD25 and CD69 on monocytes. This suggests that monocytes were in a state of cellular activation.

In the previous chapter we detected an increase in monocyte expression of CD25 and CD69 after direct stimulation with *C. pneumoniae*. However, it is unlikely that the increase in monocyte CD25 and CD69 expression after incubation with conditioned media from *C. pneumoniae* stimulated BEAS-2B cells is a result of residual bacteria. This is supported by the data obtained from whole blood that was incubated with media collected from wells in the absence of BEAS-2B cells. In this case, monocyte expression of CD25 and CD69 was expressed on a low percentage of monocytes after incubation with media alone and remained at this level after incubation with media from wells containing *C. pneumoniae* without BEAS-2B cells. This may suggest that the changes in monocyte expression of activation markers are due to secreted products from infected epithelial cells.

To determine whether IL-8 released from *C. pneumoniae* infected BEAS-2B cells played a role in the modulation of monocyte expression of CD25 and CD69, we stimulated whole blood with IL-8 but it had no significant effect on the expression of these molecules. It is

possible that other mediators, released from the epithelium are responsible for the activation of monocytes. A potential candidate for this role is GM-CSF, known to upregulate monocyte CD69 expression (447). Although there appear to be limited reports on epithelial secretion of GM-CSF in response to *C. pneumoniae* infection, *C. trachomatis* and *C. psittaci* induce GM-CSF from cervical and colonic epithelial cells (402). As the airway epithelium is a source of GM-CSF within the airways (147,165) and the levels are increased in chronic airways inflammation (137,180), it is feasible to suggest a role in monocyte activation as indicated by increased CD69 expression.

Monocyte expression of CD25 is induced by IFN-γ stimulation (460,461), but it is unlikely to be responsible for increased expression in the current studies. Airway epithelial cells are not a known source of IFN-γ. Endogenous IFN-γ secretion by whole blood leucocytes is unlikely to influence monocyte CD25 expression in the current experiments. In our studies investigating cytokine production by lymphocytes (see chapter 4), we did not detect IFN-γ expression by unstimulated lymphocytes. In addition, *C. pneumoniae* stimulation of whole blood did not stimulate lymphocyte cytokine production which eliminates the possibility that *C. pneumoniae* released from epithelial cells during the second incubation period, may have induced IFN-γ production. Furthermore, the likelihood of infectious progeny being released from infected epithelial cells during the second incubation period and stimulating whole blood leucocytes is slim. The life cycle of Chlamydia *in vitro* is reported to be completed within 72 to 96 hours (7), which is longer than the time frame of incubations used in the current methodology (a total of 48 hours).

Other mechanisms responsible for monocyte CD25 expression induced after incubation with conditioned media from *C. pneumoniae* infected BEAS-2B cells may involve bacterial antigens. Soluble chlamydial HSP-60 has been detected in airway epithelial cell supernatant from *C. pneumoniae* infected HEp-2 cells (473). Chlamydial HSP-60 is shown to activate

cells via Toll-like receptor 4 on vascular smooth muscle cells (412). Dendritic cells are also responsive to chlamydial HSP-60 but via Toll-like receptor 2 (425). Farina and colleagues show that ligands to Toll-like receptors 4 and 2 increase CD25 expression by monocytes (447). Therefore, monocyte CD25 expression observed in the current study may be mediated via HSP-60 secretion from BEAS-2B cells and subsequent activation of monocyte Toll-like receptors.

Conditioned media from BEAS-2B cells infected with WA97001 isolate caused a decrease in neutrophil CD69 expression, but direct stimulation of whole blood neutrophils with the same isolate causes a significant increase in CD69 expression (see Chapter 5). Resting neutrophils do not express CD69 (439,445) and we found that over 60% of neutrophils expressed CD69 after incubation with media from unstimulated BEAS-2B cells. In the previous chapter, only 10% of neutrophils from unstimulated whole blood cultures incubated overnight were positive for CD69. This may suggest that unstimulated BEAS-2B cells secrete a product that activates neutrophils and induces CD69 expression. It is known that GM-CSF, IFN-γ and IFN-α increase neutrophil CD69 expression (439) but of these factors, only GM-CSF is known to be expressed by airway epithelial cells (147,165). Another potential source of GM-CSF is activated monocytes. Resting monocytes do not express GM-CSF but it is induced upon activation (222,223). A substantial proportion of monocytes appear to be activated after incubation of whole blood with conditioned media from unstimulated BEAS-2B cells, as indicated by 40-60% of monocytes expressing CD69. Therefore, endogenous secretion of GM-CSF by monocytes may contribute to the high percentage of neutrophils expressing CD69 under the same conditions.

The decrease in neutrophil CD69 expression after incubation of blood with conditioned media from BEAS-2B cells infected with WA97001 ($1x10^6$ ifu/ml) is an unexpected result. A possible explanation for why these changes were not evident with conditioned media from

BEAS-2B cells infected with the IOL-207 isolate may be due to differing potencies of the isolates. We have shown in the previous chapters that epithelial cells respond to the WA97001 isolate by secreting more IL-8 and IL-6 than when infected with the IOL-207 isolate. In addition, monocytes stimulated with the WA97001 isolate express higher amounts of cytokines compared with stimulation with the IOL-207 isolate. Although, direct stimulation of blood with IL-8 did not result in a statistically significant decrease in neutrophil CD69 expression (**Table 6.7**, p=0.08), in two out of three subjects the percentage of neutrophils expressing CD69 was reduced after stimulation with IL-8. This may suggest that IL-8 may play a dual role in *C. pneumoniae* infection. The primary response of *C. pneumoniae* infected airway epithelial cells may be to increase IL-8 production, which in turn attracts neutrophils to the infection site. Continued production of IL-8 may also play a role in regulating the activation of neutrophils.

The relationship between *C. pneumoniae* infected airway epithelial cells and inflammatory cells has only been examined in terms of transmigration and cellular infiltration. The secondary effect of secreted products from infected epithelial cells to our knowledge has not previously been investigated. We have established that monocytes are activated in this situation, as indicated by increased expression of CD25 and CD69 (**Figure 6.12**). The activation status of neutrophils in terms of CD69 expression is unclear. In comparison to unstimulated neutrophils from whole blood incubated overnight, airway epithelial cells, regardless of infection status, appear to activate neutrophils and increase CD69 expression at higher *C. pneumoniae* infection titres. However, increased IL-8 production from *C. pneumoniae* infected airway epithelial cells may contribute to decreased neutrophil CD69 expression.

After assessing the activation status of monocytes and neutrophils in response to conditioned media from *C. pneumoniae* infected airway epithelial cells, we next examined the expression

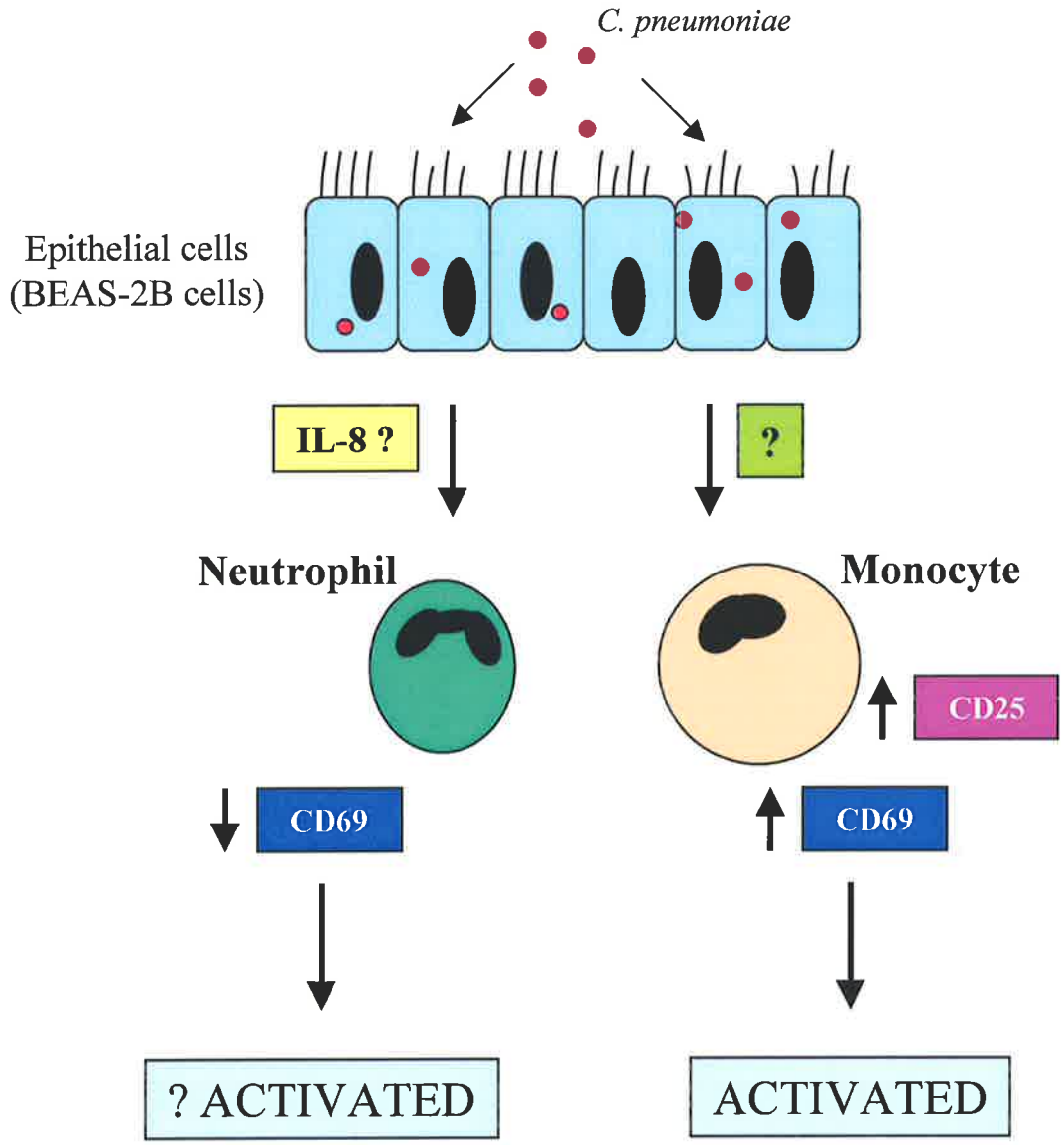


Figure 6.12: Conditioned media from *C. pneumoniae* stimulated airway epithelial cells modulates cellular activation marker expression on neutrophils and monocytes.
C. pneumoniae infected BEAS-2B cells increase secretion of chemotactic factors for neutrophils and monocytes. The release of other unknown mediators modulate CD69 and CD25 expression on neutrophils and monocytes. Media from infected epithelial cells activates monocytes as indicated by increased expression of CD25 and CD69. The activation status of neutrophils after stimulation with media from *C. pneumoniae* infected epithelial cells as we observed a decreased in CD69 expression. The contribution of IL-8 to this expression is unclear.

of leucocyte adhesion molecules. CD11b combines with CD18 to form a β_2 integrin (Mac-1) and is involved in intercellular adhesion, facilitated by the expression of its ligand, ICAM-1 expression on a range of cells (375). It is also known as complement receptor 3 (CR3) and binds opsonised particles and mediates phagocytosis (192). CD11b/CD18 is also involved in CD14 receptor mediated signalling in monocytes (424) and neutrophil functions such as chemotaxis and respiratory burst (187,491). The entire population of both monocytes and neutrophils expressed CD11b and was not changed under any of the tested conditions. However, analysis of the intensity of CD11b expression after incubation of whole blood with conditioned media from *C. pneumoniae* infected airway epithelial cells suggests a cell type specific response. We detected a significant decrease in monocyte CD11b MFI after incubation with conditioned media from *C. pneumoniae* infected BEAS-2B cells. This was more pronounced when the WA97001 isolate was used to infect BEAS-2B cells. When media was collected from wells not containing BEAS-2B cells, but incubated overnight with *C. pneumoniae*, and subsequently used to stimulate whole blood, the resulting monocyte CD11b MFI was significantly less than that observed when media was collected from BEAS-2B cells. Therefore, although there was an easily detectable level of baseline CD11b expression on monocytes, it was significantly less than that seen after incubation with conditioned media from *C. pneumoniae* infected BEAS-2B cells.

Coupled with this data, is our study investigating monocyte CD11b expression in response to direct stimulation of whole blood with *C. pneumoniae*. Monocyte CD11b MFI was significantly reduced after direct stimulation of whole blood with *C. pneumoniae*. This data is in contrast to a study performed by MacIntyre and colleagues who report an increase in monocyte CD11b expression after infection with *C. pneumoniae* (492). A possible explanation for the difference in results may be explained by the cell types utilised. We used peripheral blood and selected the monocyte population based on CD14 staining by flow cytometry. Whole blood monocytes were all positive for CD11b staining whether

unstimulated or stimulated with any of the treatments used. These results are consistent with other published studies showing constitutive expression of CD11b on peripheral blood monocytes (363,493). In contrast, the study by MacIntyre and colleagues used a monocytic cell line (THP-1 cells) and detected a low proportion of these cells expressing CD11b from uninfected cultures (492). They measured an increase in the percentage of positive cells to be approximately 75%. Although it is not possible to make a direct comparison of relative MFI units between the two studies, it appears that the sensitivity of the antibody used in our studies is higher giving an MFI of at least ten fold over that used in the study by MacIntyre and colleagues. Another cell line of myeloid lineage, HL-60 cells, do not express CD11b protein in unstimulated cultures, similar to the described THP-1 cell line, and this may be a reflection of a difference in the phenotype of monocytic cell lines and peripheral blood monocytes (492,494). Therefore, the use of peripheral blood in the current studies may be a closer representation of monocyte CD11b expression and its modulation by *C. pneumoniae*.

Decreased monocyte CD11b expression after direct stimulation of whole blood with *C. pneumoniae* may be partially due to IL-10 expression by monocytes. CD11b expression has been shown to be decreased on IL-10 stimulated monocytes and this effect is enhanced by adhesion (493). In support of this theory, we have shown that *C. pneumoniae*, particularly the WA97001 isolate, induces significant monocyte IL-10 production (see Chapter 4). We have also shown that *C. pneumoniae* stimulation of whole blood monocytes results in increased ICAM-1 expression (see Chapter 5), possibly increasing intercellular adhesion, thus potentially increasing the downregulatory effects of IL-10 on CD11b expression.

Despite this, in the studies using conditioned media as the stimulus in whole blood cultures, induction of monocyte IL-10 expression by *C. pneumoniae* released from BEAS-2B cells is doubtful. As previously discussed, due to the time frame of the experiments and the duration of the chlamydial developmental cycle, release of *C. pneumoniae* from BEAS-2B cells seems

unlikely to impact on monocyte IL-10 expression. Besides, BEAS-2B cells are not known to express IL-10 and is therefore not likely to be a potential factor secreted by these cells to cause the reduction in monocyte CD11b expression in the conditioned media studies.

Knowing that BEAS-2B cells increase IL-8 expression in response to *C. pneumoniae* infection in our studies (see Chapter 3) and others (127), we investigated the possibility that IL-8 produced by *C. pneumoniae* infected BEAS-2B cells was partly responsible for the decrease in monocyte CD11b expression. Direct stimulation of whole blood with IL-8, however, did not significantly change baseline expression of monocyte CD11b expression, thus ruling it out as a mediator in conditioned media causing reduced monocyte CD11b expression. *C. pneumoniae* infected epithelial cells also secrete IL-6 (see chapter 3)(184) and IL-6 is documented to increase monocyte CD11b expression (489). This may indicate that IL-6 release from *C. pneumoniae* infected BEAS-2B cells is a potential mediator to modulate monocyte expression of CD11b but we were unable to explore this further.

A potential consequence of decreased monocyte expression of CD11b may be a reduction in signalling via the CD14 receptor complex. In Chapter 4, we showed that chlamydial LPS may be partly responsible for changes in IL-12 and IL-1α expression. In order for monocytes to optimally respond to LPS however, CD14, CD11b/CD18 and Toll-like receptor 4 expression are required (424). Regulation of cytokine expression via this receptor complex has been demonstrated in studies by Marth and colleagues (495). Anti-CD11b and anti-CD18 monoclonal antibodies selectively downregulate monocyte IL-12 protein with no effect on TNF-α, IL-6, IL-10 or IL-1β expression (495). The reduced IL-12 may be mediated by diminished intracellular signalling generated via the CD11b/CD18 complex (424,495). Reduced CD11b/CD18 may be a pathway that is utilised by *C. pneumoniae* to decrease the efficiency with which the immune system fights infection. Not only does *C. pneumoniae* infection of epithelial cells decrease monocyte CD11b expression via soluble mediators, its

affects are also mediated by direct stimulation of monocytes by the bacteria. It has been shown that CD11b deficient macrophages have reduced IL-12 expression (424). Reduced IL-12 is also reported in states of chronic airway inflammatory disease (241-243). As chronic persistent infection with *C. pneumoniae* is associated with COPD and asthma (82,91,96,122), it is possible that the changes in IL-12 expression are mediated by reduced CD11b monocyte expression, via both *C. pneumoniae* infection of epithelial cells and direct infection of monocytes and that this may have pathophysiological relevance for the establishment of persistent infection.

A decrease in monocyte CD11b expression could be a reflection of maturation and differentiation of monocytes into macrophages. A number of studies show that CD11b expression is reduced on alveolar macrophages compared to peripheral blood monocytes (496,497). Adding to this theory, is a study performed by Yamaguchi and colleagues suggesting that *C. pneumoniae* induces the differentiation of monocytes into a phenotype associated with macrophages (255). *C. pneumoniae* infection of epithelial cells may promote migration of monocytes into the lungs which then differentiate into macrophages. An influx of macrophages are seen in chronic airways disease (177,236,237,498) which may be promoted by chronic *C. pneumoniae* infection seen in asthma and COPD (82,83,91,96,115,122).

We also examined CD11b expression on neutrophils in response to conditioned media from *C. pneumoniae* infected airway epithelial cells. Similar to monocytes, the whole population of neutrophils was stained positive for CD11b. This is consistent with published reports by other researchers illustrating constitutive expression of CD11b on over 90% of resting neutrophils (363,368). In contrast to monocytes, CD11b expression by neutrophils was significantly increased after incubation of whole blood with conditioned media from *C. pneumoniae* infected BEAS-2B cells. When media was collected from wells not

containing BEAS-2B cells, but incubated overnight with *C. pneumoniae*, and subsequently used to stimulate whole blood, the resulting neutrophil CD11b MFI was significantly less than that observed when media was collected from BEAS-2B cells. Together this data illustrates that *C. pneumoniae* infected BEAS-2B cells increase neutrophil CD11b expression which may be promoted via factors which are released from airway epithelial cells. Since IL-8 protein release is increased from *C. pneumoniae* infected BEAS-2B cells, we investigated whether it could be partly responsible for the modulation of neutrophil CD11b expression observed with the conditioned media studies. Surprisingly, direct stimulation of whole blood with IL-8 significantly reduced neutrophil CD11b MFI. Other researchers have shown that IL-8 activates neutrophils and increases chemotaxis, induces shape change an increases neutrophil respiratory burst (499,500). The activation of neutrophils is often associated with an increase in CD11b and corresponding decreased CD62L expression (364,365). In fact, stimulation of neutrophils with IL-8 is reported to increase CD11b expression (501,502). This raises the question about the viability of the neutrophils in the direct stimulation experiments described in the current studies. Although we did not directly measure the viability of neutrophils in our studies a number of findings by other researchers may suggest that neutrophil viability status may not be responsible for the observed changes in CD11b expression. Firstly, IL-8 itself is documented to inhibit neutrophil apoptosis (349,503). Secondly, neutrophils are reported to change the expression of a number of molecules when they become apoptotic. Apoptotic neutrophils decrease their expression of CD16, often with the detection of two distinct populations of neutrophils as seen with flow cytometry (504). The selection of neutrophils in the current studies were based on forward and side scatter characteristics by flow cytometry (see Chapter 2). However, to confirm that this was an appropriate gating strategy and to determine the purity of this cell population, we also stained the cells with a PE labelled CD16 antibody. We detected CD16 on the entire population of selected cells and did not observe any change in the level of expression, even with IL-8 stimulation. This illustrated that the purity of the population was high, in addition to

confirming that the viability of the cells was unaltered after incubation with a variety of stimuli. As discussed in the previous chapter, the current experimental protocol favours neutrophil survival *in vitro*. The use of whole blood cultures promotes neutrophil viability due to the presence of monocytes, reported to increase neutrophil survival (364). It is quite feasible that this culture technique may at least in part account for the differences seen in our study versus others who have stimulated purified neutrophils. It therefore seems unlikely that the decrease in neutrophil CD11b expression observed after direct stimulation of whole blood with IL-8 is attributed to cell death. Furthermore, in the experiments described herein using conditioned media from *C. pneumoniae* infected BEAS-2B cells to stimulate whole blood cultures, it appears that IL-8 does not play a role in the observed increase in neutrophil CD11b expression.

There are a number of mediators shown to upregulate neutrophil CD11b expression including TNF-α, GM-CSF and IL-6 (187,363,368,489). Epithelial cells are a known source of all these mediators in response to different stimuli (148,157,166,189,361). In earlier studies investigating the epithelial cell response to *C. pneumoniae* infection in terms of cytokine expression, we did not detect TNF-α in BEAS-2B cell supernatant (see Chapter 3), thus ruling it out as a modulator of neutrophil CD11b expression in these studies. We did not assess the expression of GM-CSF by *C. pneumoniae* infected airway epithelial cells. It is possible that endogenous GM-CSF expression by monocytes in the same cultures may contribute to increased neutrophil CD11b expression. GM-CSF is also documented to inhibit neutrophil apoptosis and inhibit reduced neutrophil CD16 expression (504,505), thus lending further support for neutrophils to be in a viable state. We did however, detect significant increases in IL-6 protein released from *C. pneumoniae* infected BEAS-2B cells and has recently been shown to increase neutrophil CD11b expression (489).

Increased CD11b expression by neutrophils is likely to be an important cellular response in an attempt to fight infection. CD11b is involved in phagocytosis, respiratory burst and chemotaxis (187,192,491). Circulating neutrophils from stable COPD patients express increased levels of CD11b in comparison to control subjects (367). Neutrophils isolated from sputum from smokers with COPD also exhibit increased CD11b expression in comparison to control subjects (371). These changes may in part be mediated by infection of the airways, both airway epithelial cells and neutrophils with *C. pneumoniae*.

As *C. pneumoniae* infection is associated with chronic respiratory disease, it is important to understand the effect that *C. pneumoniae* may have on cells actively involved in the inflammatory process. We have shown that not only does *C. pneumoniae* infection of epithelial cells decrease CD11b expression via soluble mediators, its effects are also mediated via direct stimulation of monocytes by the bacteria. This may result in a decreased ability to clear infection via reduced CD14 signalling and Th1 cytokine production, ultimately promoting persistent infection. In contrast, neutrophil CD11b expression is increased by media from *C. pneumoniae* infected epithelial cells and after direct stimulation with *C. pneumoniae*. Increased chemotaxis and phagocytosis mediated via increased CD11b expression together with continued stimulation of neutrophils via infected epithelial cells and direct infection with *C. pneumoniae* (released from epithelial cells or monocytes) may promote increased tissue inflammation and uptake of *C. pneumoniae*. Therefore, changes seen within the airways such as increased monocyte and neutrophil influx, may be regulated by *C. pneumoniae* induced changes in CD11b expression, by both direct and indirect mechanisms. The changes in leucocyte expression of CD11b as a result of stimulation with conditioned media from *C. pneumoniae* infected airway epithelial cells are summarised in **Figure 6.13**.

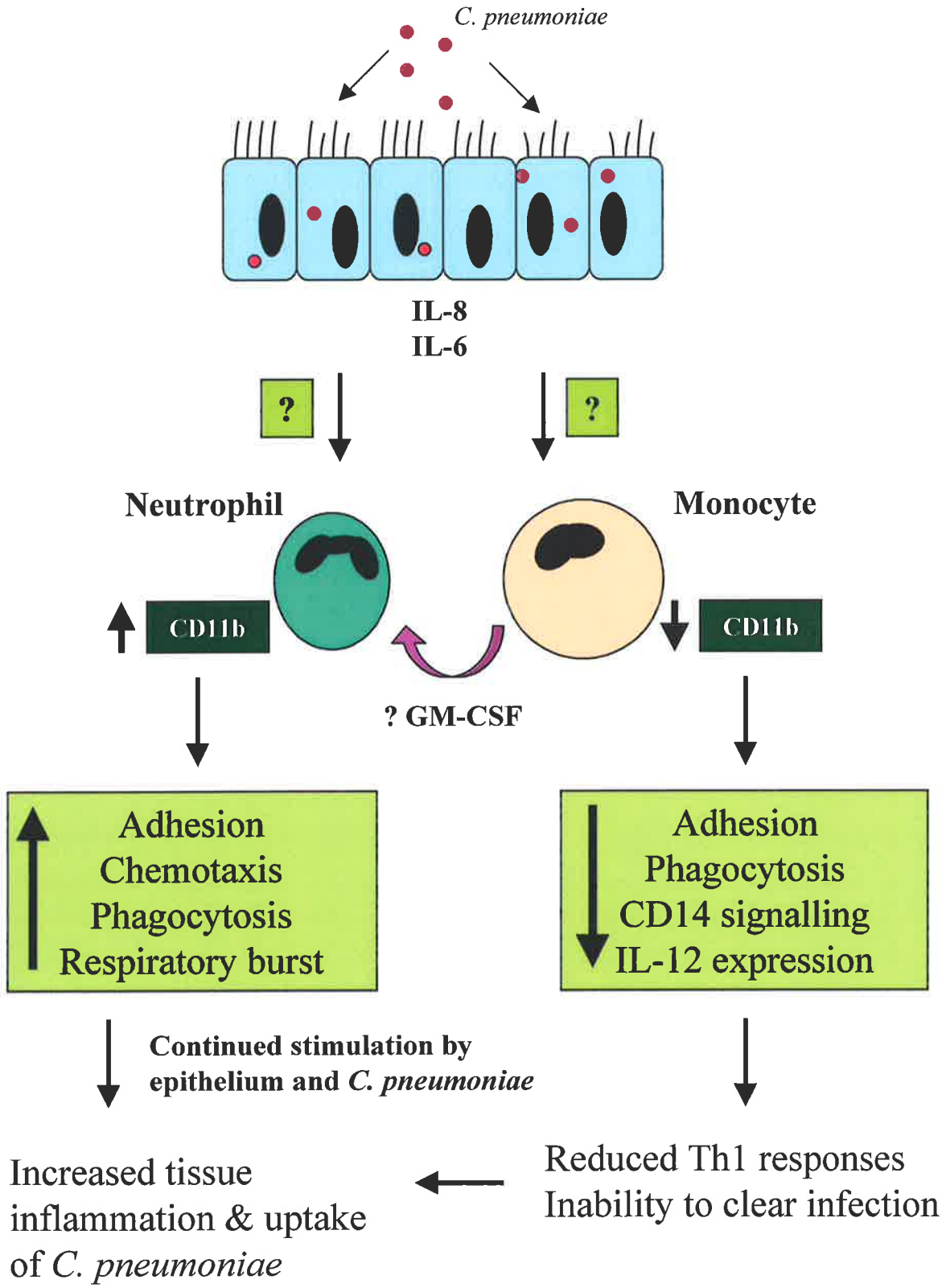


Figure 6.13: Conditioned media from *C. pneumoniae* stimulated airway epithelial cells modulates CD11b expression by neutrophils and monocytes.
C. pneumoniae infected BEAS-2B cells increase secretion of chemotactic factors for neutrophils and monocytes. The release of other unknown mediators modulate CD11b expression on neutrophils and monocytes. Endogenous GM-CSF expression by monocytes may contribute to increased neutrophil CD11b expression. Increased neutrophil expression of CD11b via *C. pneumoniae* infected epithelium or direct stimulation by *C. pneumoniae* may ultimately result in increased tissue inflammation and increased uptake of the bacteria. Decreased CD11b expression by monocytes induced by *C. pneumoniae* infected epithelium may cause diminished immune responses leading to an inability to clear primary infection.

Increased CD11b on neutrophils is often associated with a corresponding decrease in CD62L expression (364,365,372). We therefore examined CD62L expression on monocytes and neutrophils from whole blood after incubation with media from *C. pneumoniae* infected airway epithelial cells. CD62L is an adhesion molecule primarily involved in rolling adhesion between monocytes and in particular neutrophils and endothelium, playing a role in diapedesis and transendothelial migration of these cells from the circulation to sites of inflammation (506). Leucocytes lose their surface expression of CD62L via shedding of this ligand via proteolytic cleavage which occurs upon activation (363,372,488). We failed to detect CD62L on monocytes or neutrophils under any of the tested conditions. Neutrophils and monocytes from unstimulated whole blood were also negative for CD62L expression which is in contrast to previously published data (468). It is likely that the monocytes and neutrophils in the current studies were in a state of activation, partially indicated by their lack of CD62L expression. This is also indicated by constitutive expression of CD69 and CD25. Neutrophils are highly sensitive to purification and alter the expression of surface molecules but CD62L expression is maintained (373). We have used whole blood cultures to minimise these changes. Therefore, failure to detect CD62L is unlikely to be due to isolation and culture conditions. Apoptosis is also associated with loss of CD62L but as discussed the experimental protocol favours the survival of both neutrophils and monocytes.

Although the relationship between epithelial cells and *C. pneumoniae* infection has been studied in terms of leucocyte migration, no additional investigations have been performed with regard to surface molecule expression on monocytes and neutrophils as a result of *C. pneumoniae* infection of airway epithelium. Our studies suggest that the airway epithelium plays a dual role in regulating inflammatory infiltrates. Firstly, it responds to *C. pneumoniae* infection by increasing the secretion of chemotactic agents, increasing the influx of inflammatory leucocytes to the airways. Once these cells have migrated into the lungs, a

secondary effect of *C. pneumoniae* infection of airway epithelial cells may be observed. As yet undetermined factors released from the airway epithelial lining may contribute to the activation status of monocytes and neutrophils as evidenced by increased expression of cellular activation markers CD25 and CD69. Further, the expression of adhesion molecules such as CD11b are also modulated, in a cell type specific manner, with decreased monocyte expression and increased neutrophil expression of CD11b. Secondly, the lifecycle of Chlamydia permits the release of infectious progeny upon host cell lysis, thereby releasing *C. pneumoniae* organisms into the surrounding milieu of inflammatory cells which are susceptible to infection. Secondary infection of monocytes and neutrophils with *C. pneumoniae* results in a range of responses including cellular activation, as evidenced by CD25 and CD69 expression, modulation of adhesion molecule expression, not to mention the expression of a range of cytokines which are also altered. Therefore, the changes in the expression of these molecules and inflammatory mediators, known to be aberrantly expressed in chronic airways diseases, may in part be due to *C. pneumoniae* infection, which is known to be associated with these conditions.

CHAPTER 7

DISCUSSION & FUTURE DIRECTIONS

General Discussion

Since chronic airways disease such as asthma and COPD are associated with *C. pneumoniae* infection, it is important to understand how this unique bacteria may contribute to the underlying inflammation associated with these diseases. The studies documented herein have been undertaken to investigate the basic immune responses of cells susceptible to *C. pneumoniae* infection in terms of cytokine and surface marker expression profiles. By examining these responses we have also been able to highlight potential mechanisms as to how persistent *C. pneumoniae* infection may develop. The main findings of this body of work are summarised and discussed below.

The airway epithelium is the primary site of *C. pneumoniae* infection within the lungs. We therefore examined the response of airway epithelial cells to *C. pneumoniae* infection in terms of cytokine and surface marker expression. Previous studies have investigated a small range of cytokines and have focussed on IL-8 expression (127,183). By using the Cytometric Bead Array kit in these studies we examined a range of cytokines including TNF-α, IL-1β, IL-10, IL-12, IL-6 and IL-8. As documented in Chapter 3, we were able to detect significant increases in IL-8 and IL-6 secretion from a human bronchial epithelial cell line infected with *C. pneumoniae*. These studies utilised two different *C. pneumoniae* isolates that had not previously been used to examine the basic immune response of airway epithelial cells to *C. pneumoniae* infection. We found that the WA97001 isolate significantly increased cytokine expression from BEAS-2B cells, to a greater extent that that induced by the IOL-207 isolate. The increased expression of IL-8 and IL-6 from bronchial epithelial cells in COPD and asthmatic patients (137,164,165,167,168) is similar to the increased expression we have shown here with direct stimulation with *C. pneumoniae*. Given that a significant percentage

of these patients are known to have *C. pneumoniae* infection, the increased expression of inflammatory mediators from bronchial epithelial cells may in part be mediated by *C. pneumoniae* (**Figure 7.1**).

The induction of inflammatory mediators from *C. pneumoniae* infected epithelial cells, in particular IL-8, are shown to be involved in the recruitment of inflammatory cells and transepithelial migration (127,356). In addition, animal models of *C. pneumoniae* infection result in infiltration of inflammatory cells to the initial site of infection (146,357). As *C. pneumoniae* is an intracellular bacterium, it grows and divides within the host cell. Upon completion of its developmental cycle, host cells are lysed and infectious *C. pneumoniae* progeny are released enabling further infection of neighbouring epithelial cells or infection of cells recruited to the inflamed tissue. Therefore, we investigated the response of leucocytes, which may be recruited to the site of infection, to direct stimulation of *C. pneumoniae*, which may be released from bronchial epithelial cells (**Figure 7.2**).

The studies described in Chapter 4 examine the response of whole blood leucocytes to *C. pneumoniae* stimulation in terms of cytokine production. Previous studies investigating *C. pneumoniae* modulation of monocyte cytokine production have used monocytic cell lines (215,252) or peripheral blood mononuclear cell preparations without further purification of leucocyte cell subsets (249-251). By using flow cytometry we have been able to confidently identify leucocyte subsets and identify intracellular cytokine production by monocytes and lymphocytes. However, under the current experimental conditions, we did not detect any lymphocyte cytokine expression induced by *C. pneumoniae*. The basic immune response of monocytes to *C. pneumoniae* stimulation involved the induction of a range of cytokines including TNF-α, IL-1α, IL-6, IL-8, IL-10 and IL-12. The increased expression of these cytokines by monocytes stimulated with *C. pneumoniae* may sustain and promote

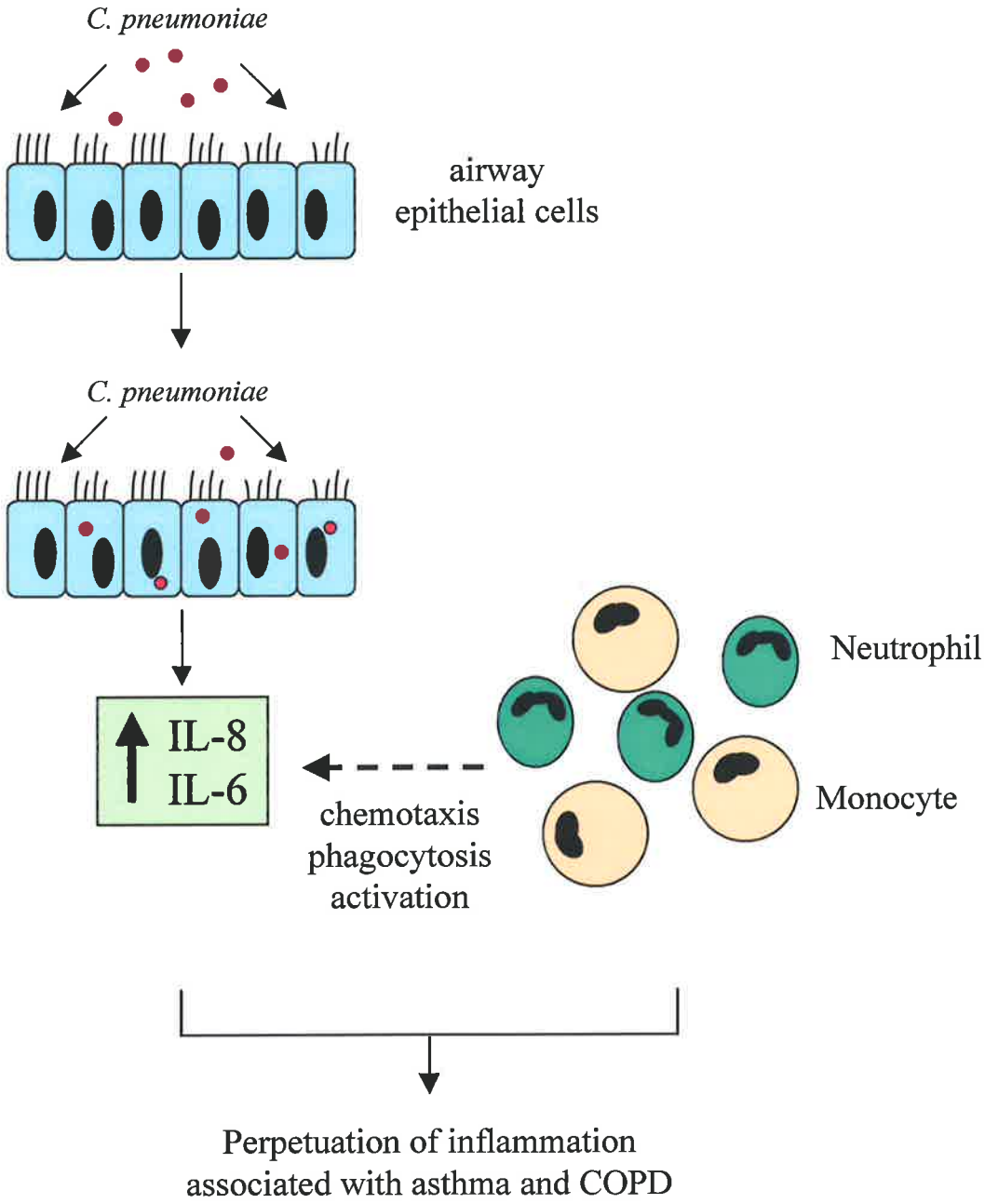


Figure 7.1: *C. pneumoniae* increases IL-8 and IL-6 secretion by airway epithelial cells.
Infection of airway epithelial cells with *C. pneumoniae* induces the release of mediators that are chemotactic for leucocytes. *C. pneumoniae* induced cytokine production within the airways and infiltration of inflammatory cells may exacerbate asthma and COPD by perpetuating inflammatory processes.

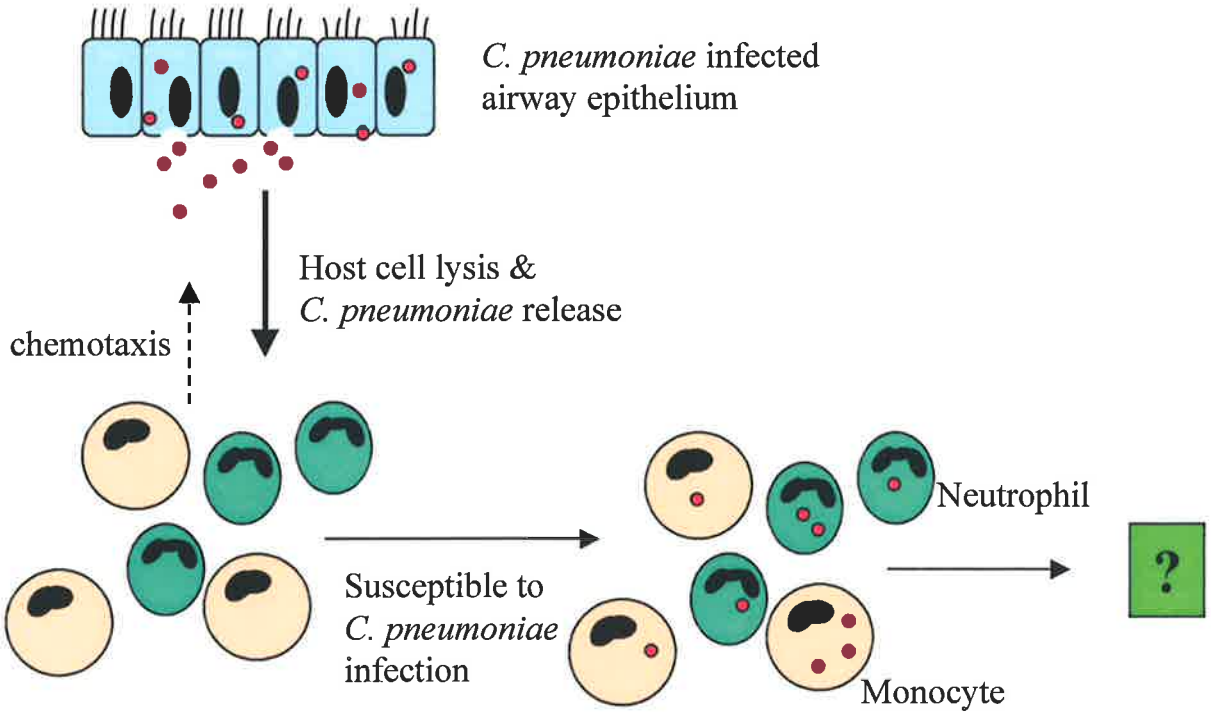


Figure 7.2: Sequence of events leading to leucocyte infection with *C. pneumoniae*.
C. pneumoniae infected airway epithelial cells release chemotactic factors, attracting leucocytes to the site of infection. On completion of the developmental cycle of *C. pneumoniae*, host cells (airway epithelium) lyse releasing infectious progeny which are able to infect susceptible infiltrating leucocytes. We therefore investigated the host cell-pathogen relationship by examining the response of leucocytes to *C. pneumoniae* infection.

inflammation seen in respiratory disease which is now known to be associated with chronic *C. pneumoniae* infection.

It has been suggested that persistent chlamydial infection may develop as a result of an imbalance between Th1 and Th2 type responses (304-306,423). Although these classifications usually refer to lymphocyte responses, IL-12 and IL-10 are characterised as Th1 and Th2 type cytokines respectively. In this respect, we further investigated monocyte expression of IL-12 and IL-10 in response to *C. pneumoniae*. We observed that with increasing concentrations of *C. pneumoniae*, IL-12 expression had a tendency to decrease, which was not attributed to cell death. In addition, increased concentrations of *C. pneumoniae*, particularly the WA97001 isolate, caused a dose response relationship with regard to monocyte expression of IL-10.

The clinical implications of the imbalance between IL-12 and IL-10 monocyte expression as a result of *C. pneumoniae* stimulation may be two fold. Firstly, Th1 responses are required for lymphocyte proliferation and activation and cytotoxicity of T cells and NK cells. Monocyte production of IL-12 is known to regulate these processes (289). Therefore, promotion of Th1 type responses are associated with cell mediated immunity and clearance of infection. However, the decrease in Th1 responses observed in the current experiments as evidenced by decreased IL-12 expression, and corresponding increase in Th2 type responses, as evidenced by increased IL-10 expression by *C. pneumoniae* stimulated monocytes, may result in a net increase in Th2 responses. This shift in the balance between IL-12 and IL-10 expression may decrease cell mediated immunity by reducing T cell proliferation and activation together with an inhibition of T cell and NK cell cytotoxicity, overall resulting in the promotion of Th2 type responses. An inability to clear infection due to reduced cell mediated immunity may therefore, eventually lead to the development of chronic or persistent *C. pneumoniae* infection that is commonly associated with COPD and asthma (82,91,96,122).

Secondly, the pattern of inflammation associated with asthma is described as a Th2 type response. This is supported by increased production of Th2 cytokines including IL-10, IL-4 and IL-5 (135,166,507). A corresponding decrease in IFN-γ and IL-12 expression correlating to decreased Th1 cellular immune responses is also evident (242,243). Therefore, the pattern of monocyte cytokine expression induced by *C. pneumoniae* as seen here, with regard to IL-10 and IL-12 expression is similar to the patterns associated with asthma. Since asthma is associated with *C. pneumoniae* infection, the cytokine profile of monocytes observed in asthmatic patients may in part be mediated by *C. pneumoniae* infection.

In the chapter of work investigating cytokine production by monocytes stimulated with *C. pneumoniae*, we were able to extend our findings to include a study of potential chlamydial antigens responsible for cytokine induction. We demonstrated that *C. pneumoniae* antigens may regulate monocyte cytokine production via cytokine specific mechanisms. *C. pneumoniae* induced monocyte expression of IL-1α appeared to be mediated via multiple antigens. In contrast, IL-12 expression seemed to be predominantly induced by a heat stable chlamydial protein. The chlamydial antigens that are most likely to play a role in monocyte stimulation are chlamydial LPS and chlamydial heat shock proteins. Chlamydial LPS is similar in structure to endotoxins from other bacteria, although its potency is significantly less (508). LPS mediates a variety of monocyte responses via surface expression of CD14 and Toll-like receptors (424,477,478). LPS stimulation of monocytes causes increased expression of TNF-α, IL-1, IL-6, IL-8, IL-12 and IL-10(223,226). The expression of these cytokines is modulated in COPD and asthma, both of which are associated with *C. pneumoniae* infection. Therefore, the pattern of cytokine expression in chronic respiratory disease may in part be mediated by chlamydial LPS.

As discussed in Chapter 4, chlamydial HSP-60 is unlikely to be involved in IL-1α expression. However, it may play a role in the modulation of other cytokines. A variety of cells respond to chlamydial HSP-60 by increasing production of TNF-α, IL-6, IL-8 (253,254,349). During chronic or persistent *C. pneumoniae* infection, known to be associated with chronic respiratory disease, HSP-60 continues to be expressed (70,473,509) and may promote inflammatory responses similar to those observed in the current studies. Chlamydial HSP-60 is shown to be significantly associated with asthma (121) and indicators of lung function are inversely correlated to chlamydial HSP-60 titres suggesting an association with disease severity (121).

In conclusion, the studies assessing the response of peripheral blood monocytes to *C. pneumoniae* stimulation suggest that induction of monocyte cytokines such as TNF-α, IL-1α, IL-6 and IL-8 may contribute to and sustain the inflammation observed in chronic airways disease (**Figure 7.3A**). Various chlamydial antigens, some of which are associated with lung disease, may mediate these profiles. In addition, increased *C. pneumoniae* stimulation of monocytes promotes Th2 type cytokine profiles that may lead to persistent infection (**Figure 7.3B**).

In addition to cytokines, the expression of cell surface molecules play an important role in the regulation of inflammatory processes. Three main processes mediated by surface molecules are required for optimal activation of T lymphocytes, adhesion, antigen presentation and costimulation. In Chapters 5 and 6 we investigated the expression of surface molecules on leucocytes involved in these cellular processes in response to direct *C. pneumoniae* stimulation and indirect stimulation via *C. pneumoniae* infected airway epithelial cells. In both cases the response of leucocytes to *C. pneumoniae* infection in terms of surface molecule expression, predominantly involved monocytes and neutrophils. Using CD25 and CD69 as markers of cellular activation, we found that monocytes and neutrophils were activated by

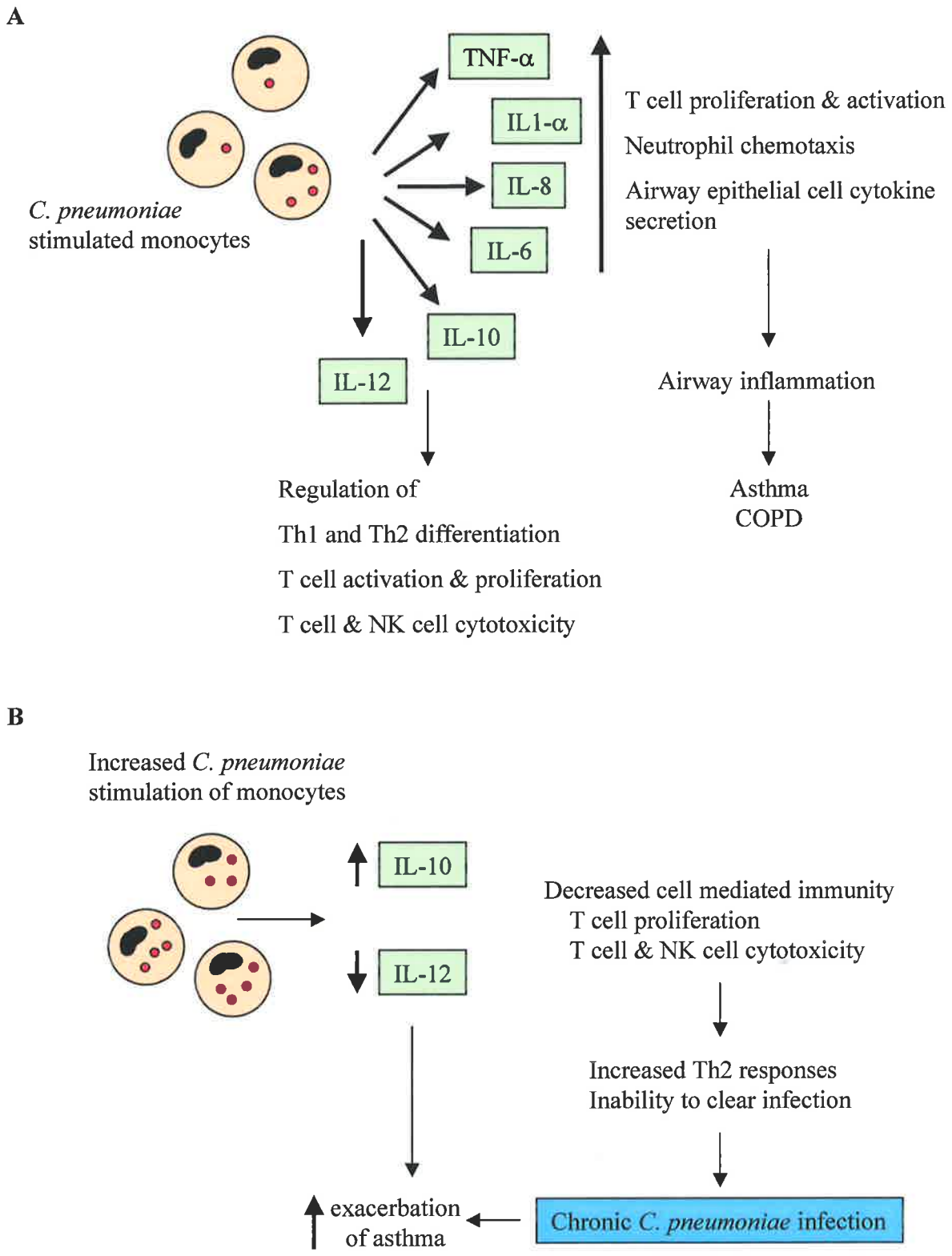


Figure 7.3: *C. pneumoniae* modulates whole blood monocyte cytokine expression which may lead to persistent infection.
A) Whole blood monocytes stimulated with *C. pneumoniae* increase the production of a range of cytokines. These mediators are involved in the regulation of cell mediated immunity. Increased activity of lymphocytes, airway epithelial cells and neutrophils are observed in chronic lung diseases. COPD and asthma are associated with *C. pneumoniae* infection, which may in part be responsible for inflammation associated with these disease states. **B)** Increased stimulation of monocytes with *C. pneumoniae* results in increased IL-10 (Th2) and decreased IL-12 (Th1) expression, skewing immune responses toward a Th2 type pattern. This may lead to an inability to clear primary *C. pneumoniae* infection, allowing chronic infection to develop which is associated with chronic lung disease.

direct stimulation with *C. pneumoniae* and by mediators released from *C. pneumoniae* infected airway epithelial cells (**Figure 7.4**). Lymphocytes appeared to be activated but to a lesser extent with only minimal induction of CD25 expression after exposure to *C. pneumoniae*. A third surface marker, CD11b is also linked to neutrophil activation (187,489,491). Neutrophil CD11b expression was increased after incubation with conditioned media from *C. pneumoniae* infected airway epithelial cells providing additional evidence of neutrophil activation. Clinical samples from patients with chronic lung disease also show increased cellular activation, as evidenced by increased CD25, CD69 or CD11b on a variety of cell types (237,314,367,371). The expression of surface molecules associated with cellular activation induced by *C. pneumoniae* observed in the current experiments, is similar to that seen in chronic lung disease (**Figure 7.4**). Since it is now known that diseases such as asthma and COPD are significantly associated with *C. pneumoniae* infection, it is possible that increased cellular activation in these disease states may in part be attributed to *C. pneumoniae* infection.

Included in the same studies were a series of experiments examining the expression of a range of leucocyte adhesion molecules in response to *C. pneumoniae*. The main findings were associated with increased ICAM-1 expression on monocytes. This is consistent with previous publications using monocytic cell lines (252,255). This increase may facilitate adhesion of monocytes and neutrophils in cellular aggregation with cells expressing LFA-1 including lymphocytes. Most studies investigating ICAM-1 expression in the airways has focussed on bronchial epithelial cell ICAM-1 expression (191). However, there is some evidence that ICAM-1 is increased on inflammatory cells from asthmatics and increased circulating levels of soluble ICAM-1 (313,314). Neutrophils also responded to *C. pneumoniae* by decreasing a second adhesion molecule, CD58 (LFA-3). The counter receptor for CD58 is CD2, which is exclusively expressed on lymphocytes. *C. pneumoniae* modulation of CD58 on neutrophils may therefore, impact on interactions between neutrophils and lymphocytes. Ligation

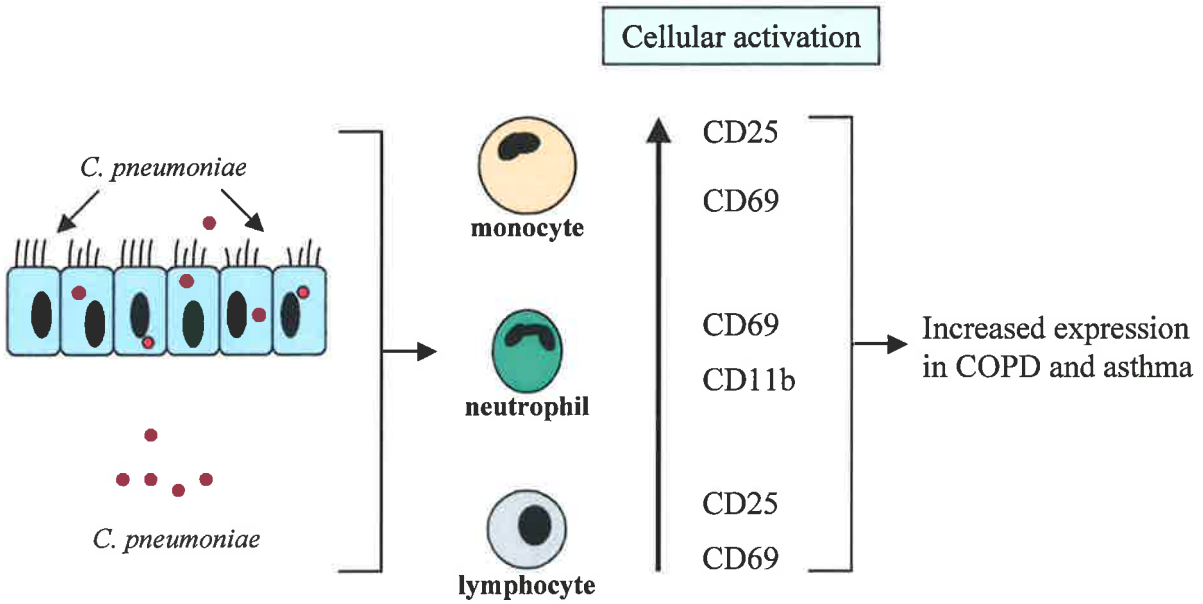


Figure 7.4: *C. pneumoniae* activates leucocytes as evidenced by increased surface molecule expression. Exposure of leucocytes to conditioned media from *C. pneumoniae* infected airway epithelial cells, or direct stimulation with *C. pneumoniae* results in increased expression of cellular activation molecules. These molecules are also increased in chronic lung disease which is also associated with *C. pneumoniae* infection. Therefore, *C. pneumoniae* may contribute to the activation of inflammatory cells in chronic lung disease.

between these two molecules facilitates adhesion and generates intracellular signalling pathways in lymphocytes. Therefore, decreased neutrophil CD58 expression may diminish the immune response to *C. pneumoniae* in two ways. Firstly by decreasing the physical association between neutrophils and lymphocytes and thus reducing the delivery of costimulatory signals.

However, one of the most striking responses of leucocytes to *C. pneumoniae* stimulation involved monocyte expression of the B7 family of costimulatory molecules CD80 and CD86. These molecules are suggested to be representative of Th1 and Th2 responses respectively (276,475). Since the data generated from experiments examining *C. pneumoniae* induced monocyte cytokine production showed a tendency to decrease Th1 (IL-12) and increase Th2 (IL-10) resulting in an overall increase in Th2 responses, we were interested to see if similar changes were observed with respect to surface molecule expression. However, we found that *C. pneumoniae* induced only low expression of CD80 (Th1) and significantly reduced CD86 (Th2) expression on monocytes. Therefore, although the pattern of monocyte costimulatory molecule expression induced by *C. pneumoniae* appeared not to be dominated by Th1 or Th2 responses, the reduction in costimulatory signals may be evident of a more generalised response of decreased activation of T lymphocytes (**Figure 7.5**). IL-10 is shown to inhibit CD80 expression (269) which may be a factor in the low induction of CD80 expression in the current experiments. There is evidence in the literature that suggests that the provision of costimulatory signals to lymphocytes are reduced in chronic airways disease (272,510). The response pattern and expression of CD80 and CD86 seen in COPD and asthma are similar to those that we have observed after direct stimulation of cells with *C. pneumoniae*. Given that a high percentage of these patients are now known to have evidence of chronic *C. pneumoniae* infection, the pattern seen could in part be mediated by *C. pneumoniae*. As discussed in Chapter 5, inadequate activation of T cells may lead to reduced cell mediated immunity and

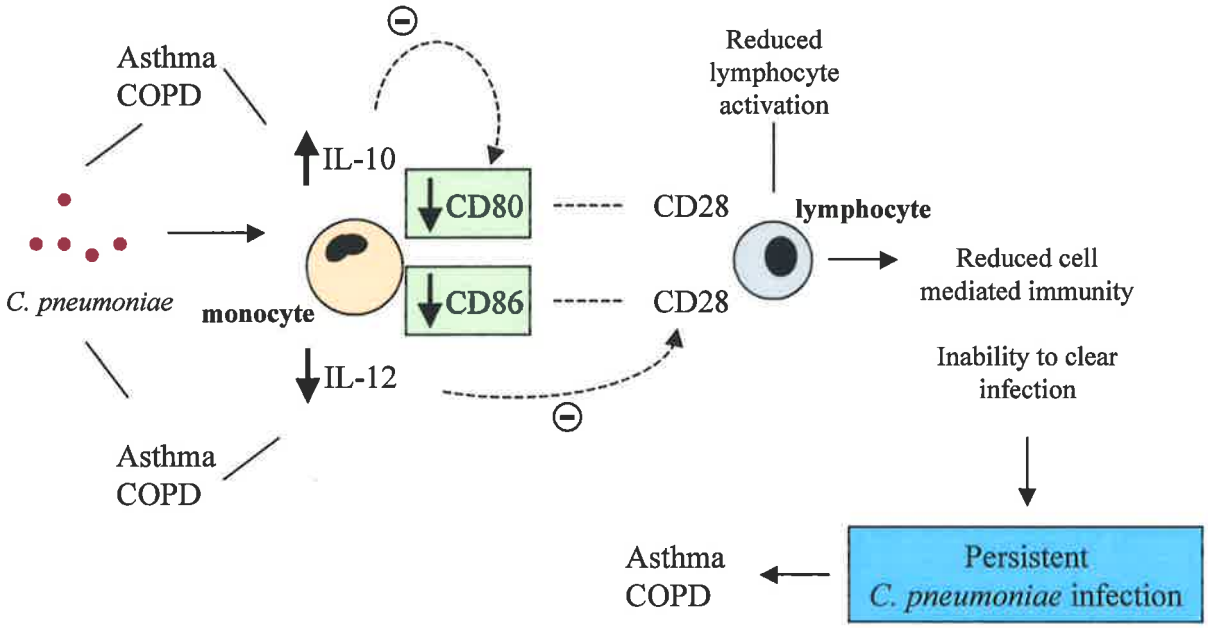


Figure 7.5: *C. pneumoniae* decreases monocyte costimulatory molecule expression.
Monocyte expression of costimulatory molecules, CD80 and CD86 is decreased. Increased IL-10 expression may play a role in reduced CD80 expression. At high concentrations of *C. pneumoniae*, reduced IL-12 may decrease synergism with CD28 signalling. Diminished costimulatory molecule expression by *C. pneumoniae* stimulated monocytes may reduce lymphocyte activation leading to reduced cell mediated immunity and ability to clear infection. This may promote the development of persistent *C. pneumoniae* infection exacerbating chronic inflammation in asthma and COPD.

an inability to clear acute infection. This would potentially allow *C. pneumoniae* to establish persistent infection, which is associated with chronic lung disease.

In summary, we have undertaken a comprehensive study investigating the relationship between *C. pneumoniae* and host cells. As described in **Figure 7.6**, the airway epithelium may play an important role in regulating the immune response to *C. pneumoniae* infection. These cells release IL-6 and IL-8 in response to infection, as well as other unknown factors that may impact on the expression of both surface molecules and cytokines expressed by leucocytes. Release of *C. pneumoniae* from lysis of airway epithelial cells may then provide an opportunity for infectious progeny to infect infiltrating leucocytes. The response of leucocytes to *C. pneumoniae* predominantly involved monocytes and neutrophils. Monocytes are activated as evidenced by *C. pneumoniae* induced CD25 and CD69 expression. *C. pneumoniae* activated monocytes increase the production of a variety of cytokines but the overall balance may be skewed toward Th2 type patterns with increased IL-10 and decreased IL-12 expression in the presence of hight concentrations of chlamydia. As yet undefined mediators released from *C. pneumoniae* infected airway epithelial cells decrease monocyte CD11b expression and may regulate IL-12 secretion. This may promote a similar pattern of inflammation associated with chronic lung disease and a reduced ability to clear infection. In addition, costimulatory signals provided by monocytes to lymphocytes are reduced via decreased CD80 and CD86 expression. An increase in IL-10 expression could be a potential factor involved in only minimal induction of CD80 expression. Furthermore, a reduction in IL-12 may contribute to reduced synergism usually occurring on the activation of the B7/CD28 pathway. Combining the two pathways of Th2 cytokine expression and reduced costimulation via CD80 and CD86 expression may lead to inadequate T lymphocyte activation and proliferation. A reduction in these processes would potentially reduce cell mediated immunity via IFN-γ and IL-2 expression leading to an inability to clear acute *C. pneumoniae* infection, thus leading to chronic or persistent infection.

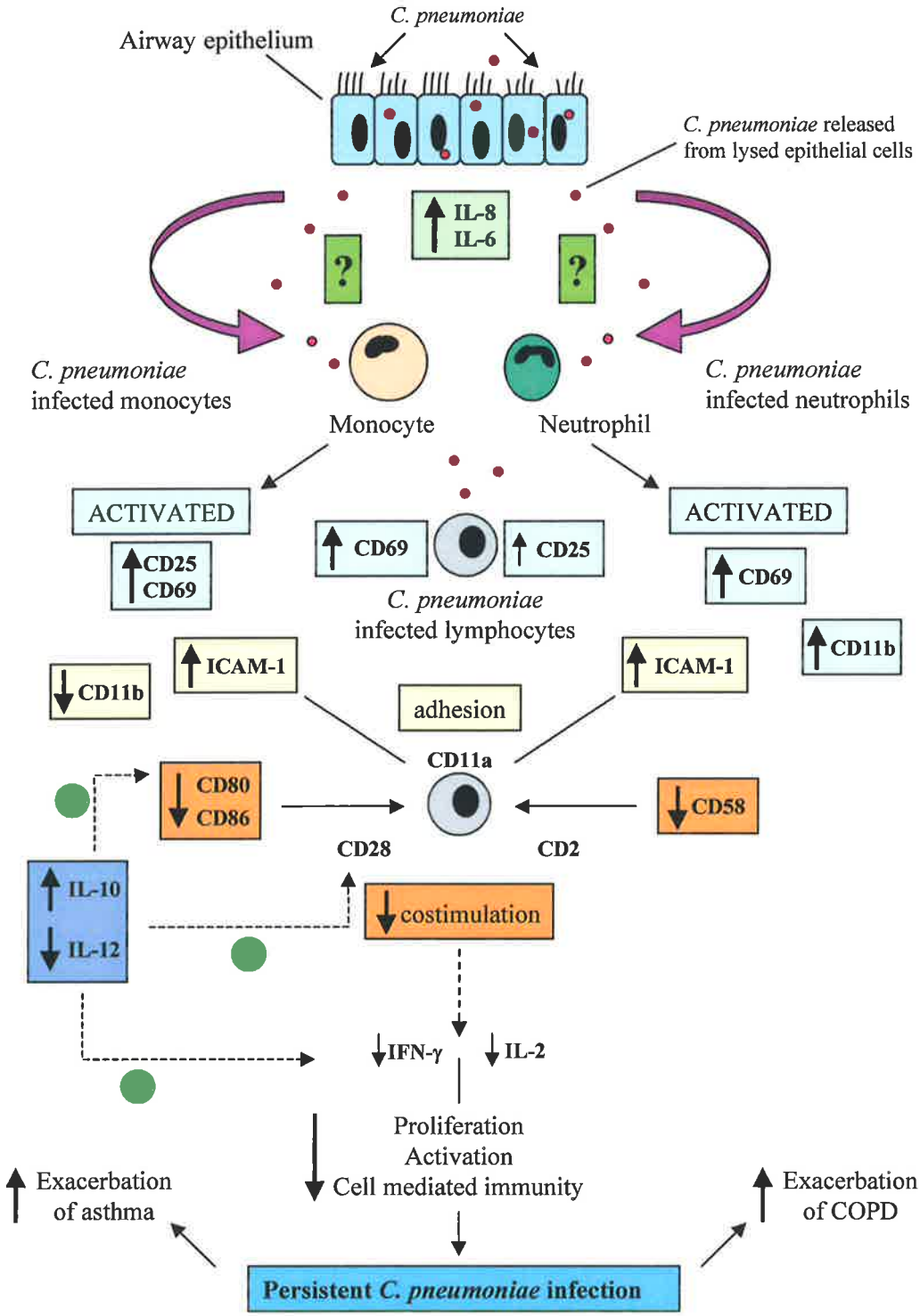


Figure 7.6: Proposed model of the development of persistent *C. pneumoniae* infection associated with airways inflammation.
Airway epithelial cells are susceptible to *C. pneumoniae* infection and increase the production of IL-8, IL-6 and as yet unknown factors. These factors play a role in the recruitment of inflammatory cells to the site of infection where they may also become infection with *C. pneumoniae*. Monocytes, neutrophils and to a lesser extent lymphocytes are activated by *C. pneumoniae*, increasing the expression of cellular activation markers. Monocytes also respond to infection by modulating adhesion molecule expression, altering their interaction with other leucocytes. Some of these molecules may be involved in the observed decrease in Th1 cytokines (IL-12) and increase in Th2 (IL-10) cytokines in response to *C. pneumoniae* stimulation. Monocytes also decrease their expression of B7 molecules involved in the costimulation of lymphocytes, which may be further compounded by reduced IL-12 expression. Exposure of neutrophils to *C. pneumoniae* causes modulation of adhesion molecules which may impact on their interactions with lymphocytes, decreasing adhesion and costimulation. Decreased activation of T cells and cell mediated immunity may lead to the development of persistent *C. pneumoniae* infection. The development of persistent infection may lead to exacerbation of chronic lung disease. Therefore, *C. pneumoniae* may promote its own persistent infection which is associated with chronic lung disease and may in part be responsible for the observed inflammation in these disease states.

The response of neutrophils to acute *C. pneumoniae* infection may also impact on the ability of lymphocytes to mount an efficient immune response. Since IL-8 is a potent chemoattractant for neutrophils, its expression by *C. pneumoniae* infected airway epithelial cells would promote neutrophil migration to the airways (**Figure 7.6**). The release of infectious progeny from airway epithelial cells may then infect infiltrating neutrophils. The response of neutrophils to *C. pneumoniae* include increased surface expression of CD69 and CD11b, both of which are indicators of neutrophil activation. Increased CD11b and ICAM-1 may facilitate increased adhesion with lymphocytes. Since neutrophils do not commonly express molecules from the B7 family (CD80, CD86), ligation of CD58 and CD2 may provide an alternative costimulation pathway. However, *C. pneumoniae* decreases neutrophil CD58 expression, which may reduce the ability of neutrophils to provide costimulatory signals to lymphocytes. Together with reduced monocyte signals via the B7/CD28 pathway, the ability of lymphocytes to respond to acute *C. pneumoniae* infection may be compromised.

The ability of *C. pneumoniae* to reduce the expression of surface molecules that are essential to providing lymphocytes with costimulatory signals, may be a potential mechanism that promotes the development of persistent infection. It is becoming more apparent that there is an association between chronic *C. pneumoniae* infection and chronic lung disease. It is therefore important to gain an understanding how persistent infection is established. An understanding of the potential mechanisms promoting the development of chronic *C. pneumoniae* infection may provide new opportunities for therapeutic intervention.

Future Directions

In the current studies we have successfully developed a model of *C. pneumoniae* infection of airway epithelium. The BEAS-2B cell line has proven to be a valuable tool to identify the response of airway epithelium to *C. pneumoniae* infection. A more detailed analysis of mediators released from the airway epithelium in response to *C. pneumoniae* infection however is still required. In particular, the effect of *C. pneumoniae* infection on the expression of GM-CSF and MCP-1 would increasing our understanding of how the airway epithelium regulates and orchestrates downstream inflammatory processes.

We have also developed a second model of *C. pneumoniae* infection and utilised flow cytometry to examine intracellular cytokines and cell surface molecules in leucocytes. To gain a closer representation of the inflammatory patterns induced by *C. pneumoniae* in the respiratory system, these models need to be applied to clinical respiratory specimens. Collection of peripheral blood and bronchoalveolar lavage samples from well characterised groups of patients diagnosed with asthma, COPD and other chronic respiratory diseases, together with their current *C. pneumoniae* infection status and would enable a comprehensive study of the role of *C. pneumoniae* infection in airways inflammation to be performed. Based on the results of the current research, particular emphasis and focus on costimulation of T cells would provide a better understanding of how *C. pneumoniae* may promote persistent infection. Identification of mechanisms involved in activation of T cells, which are disrupted by *C. pneumoniae* may also allow for the development of new therapies to restore the balance of the immune system to adequately fight and clear infection.

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