Response of Endothelial Cells to exposure to *Chlamydia trachomatis*, biovar LGV.

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Submitted in fulfilment of the requirements for the degree of Master of Medical Science (Medical Microbiology) in the Department of Infection Prevention and Control

As the candidate’s supervisor I agree to the submission of this dissertation.

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PRESENTATIONS FROM THIS THESIS

I.D. Seipone and A.W. Sturm. 2008. Comparison of the effect of *Chlamydia trachomatis* strains (Lymphogranuloma Venereum and Ocular genital) in/on endothelial cells.

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DEDICATION

This dissertation is dedicated to my dad, Kgosi C.P. Seipone. I am where I am because of the way you raised me up. Thanks for the values and morals you instilled in me and raising me up to believe that I can be everything that I want to be. May God grant you many more years.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ANOVA	analysis of variance
Bcl-2	B-cell lymphocytic – leukaemia proto-oncogene 2 protein
BH3	Bcl-2 homolog domain -3
Bik	Bcl-2-interacting killer
Bim	Bisindolylmaleimide
BSA	bovine serum albumin
BSS	buffered saline solution
CAMs	cellular adhesion molecules
CGM	Chlamydia growth medium
C. trachomatis	*Chlamydia trachomatis*
C. pneumoniae	*Chlamydia pneumoniae*
C. psittaci	*Chlamydia psittaci*
CO_2	carbon dioxide
CDC	Center for Disease Control and Prevention
DAB	diaminobenzidine
DAP	2.4-di-amino-6-pyrimidine
DNA	deoxyribonucleic acid
DFA	direct immuno-fluorescence antibody
DPX	distyrene plasticizer xylene
EB	elementary body
EDTA	ethylenediaminetetraacetic acid

EIA	enzyme immunoassays
EGM	endothelial growth medium
ELR	Glu-Leu-Arg
EMEM	eagle's minimum essential medium
ELISA	enzyme- linked immunosorbent assay
FBS	foetal bovine serum
FCS	fetal calf serum
FITC	flourescein isothiocynate
HBSS -	hanks' balanced salt solution
HEPES -	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP -	horseradish peroxidase
HMIEC	human intestinal microvascular endothelial cell
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular cell adhesion molecule
IFU	inclusion forming unit
IL	interleukin
LDH	lactate dehydrogenase
LFA - 1	leukocyte function-associated antigen-1
LGV	Lymphogranuloma Venereum
MAb	monoclonal antibody
Mac-1	macrophage-1 antigen
MCP-1	monocyte chemotactic protein-1
MOI	multiplicity of infection
MOMP	major outer membrane protein
NAD	nicotinamide adenine dinucleotide

OG	Oculogenital
ORF	Open Reading Frame
PAF	platelet activating factor
PARP	poly (DAP-ribose) polymerase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline (pH 7.4)
PID	pelvic inflammatory disease
PMEC	polyp microvascular endothelial cell
PS	phosphatidylserine
Puma	p53 upregulated modulator of apoptosis
R^3 – IGF- 1	recombinant long R insulin –like growth factor-1
RB	reticulate body
rTdT	recombinant terminal deoxynucleotidyl transferase
rhEGF	recombinant human endothelial cell growth factor
rhFGF-B	recombinant human fibroblast growth factor-B
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
sICAM	soluble intercellular adhesion molecule
SPG	sucrose glutamic buffer (pH 7.5)
spp	species
SSC	Sodium-Saline Citrate
TMB	tetramethylbenzidine
TEM	transendothelial migration
TNFα	tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end

	labeling
UV	ultraviolet
VAD-FMK	Val-Ala-Asp-α-fluoromethylketone
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WHO	World Health Organization

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ETHICS

This study was approved by the Biomedical Ethics Committee of the University of Kwa-Zulu Natal (BF060/07), approval to use the stored isolates was under reference number H184/04.

ABSTRACT

Although both are caused by *Chlamydia trachomatis*, Lymphogranuloma Venereum (LGV) presents differently from the infections caused by Oculogenital (OG) strains. The endothelium of blood and lymph vessels allows passage of cells to the site of infection. Endothelial cells also secrete chemokines and cell adhesion molecules which act as attractants and binding sites for various cellular immune components. Since LGV biovar affect the lymphoid tissue we studied the effect of *C. trachomatis* on endothelial cells.

Human umbilical vein endothelial cells (HUVEC) were infected with *C. trachomatis* LGV serovars L1, L2, L3 and the OG strain E at multiplicity of infection (MOI) of 1 and incubated for 24 hours. Stimulation of Interleukin-8 (IL-8) and monocyte chemokine protein-1 (MCP-1) chemokines and the intercellular adhesion molecule -1 (ICAM-1) were quantified by enzyme linked immunosorbent assays (ELISA). Transendothelial migration of neutrophils and monocytes was carried out in transwells. The lactate dehydrogenase (LDH) release assay was used to measure cell necrosis. Apoptotic cell death was analysed using the BioVision^{TM} CaspGLOW Fluorescein Caspase Staining Kit and DeadEnd^{TM} Colorimetric Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) system with the C. *trachomatis* Culture Confirmation kit as a counter stain.

All *Chlamydia trachomatis* serovars (L1, L2, L3 and E) successfully infected and replicated in HUVEC after 24 hours of infection. Only L3 stimulated significantly higher production of IL-8, MCP-1 and ICAM-1 by HUVEC as compared to the

negative control and mock-infected cells. However, the remaining LGV serovars (L1 and L2) and the OG serovar E showed no significant difference in the stimulation of IL-8, MCP-1 and ICAM-1 when compared to the controls. Comparison of LGV and OG serovars showed no significant difference between these two biovars in inducing production of IL-8 and MCP-1, but L3 stimulated ICAM-1 at a significantly higher level than E. There was no significant difference in the number of migrated neutrophils between untreated HUVEC, mock infected HUVEC and HUVEC infected with Chlamydia serovars. L2 and L3 had significally higher amount of migrated monocytes than the controls with L3 being the highest. L3 was the only serovar that had a significant level of cell death by necrosis. Apototic cells were observed in both uninfected and infected HUVEC which is due to normal cell turn over. None of the infected cells showed TUNEL positive nuclei.

It can be concluded that L3 is more virulent than the other serovars during the first 24 hours of infection. Infection with *C. trachomatis* serovars does not seem to cause any cell death by apoptosis 24 hours post infection. The only cell death that occurs is by necrosis and only on serovar L3 infected cells.

CHAPTER 1: INTRODUCTION

Lymphogranuloma Venereum (LGV) serovars have been shown to be more virulent than the Oculogenital (OG) strains (Mabey and Peeling, 2002). Not much is known about differences in pathogenesis of LGV and oculogenital disease. Lymphogranuloma venereum serovars pass through the epithelial lining and seem to primarily infect macrophages that migrate to the regional lymph nodes causing disseminated infection. The OG biovars however infect the mucosal epithelial surface of the eye and genital tract and are mainly confined to that position (Mabey and Peeling, 2002). This study focuses on endothelial cells since they line the lumina of lymphatic vessels which are used for migration into the lymphnodes. The endothelium plays a crucial role in immunity in that recruited immune cells have to pass through it in a process known as extravasation to get to the point of infection; they also secrete mediators such as chemokines and cell adhesion molecules which act as attractants and binding sites for various cellular immune components facilitating diapedesis (Farjado 1989).

Chlamydia species has been shown to induce the production pro-inflammatory molecules (chemokines and adhesion molecules) on the infected host cell. These in turn play a role in recruiting leukocytes to the site of infection (Stephens, 2003). Interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) chemokines recruit neutrophils and monocytes respectively to the site of infection, which are the main cells involved in the initial phase of fighting infections. There has not been much work done in comparing the different serovars in terms of stimulation of chemokine

production by the infected cell. Most of the studies focus on *Chlamydia pneumoniae* without comparison between strains or biovars.

Furthermore, there has been some contradicting reports regarding *Chlamydia trachomatis* and apoptosis with some research showing that Chlamydia is anti-apoptotic and others that Chlamydia infection does induce apoptosis. Fan *et al.* (1998), Dean and Powers (2001), Fischer *et al.* (2004), Dong *et al.* (2005) and Ying *et al.* (2005) have done studies which support the anti- apoptotic characteristics of *C. trachomatis*. In contrast, studies done by Ojcius *et al.* (1998), Jean-Luc *et al.* (1999) and Samuel and Stephens (2001), stipulate that *C. trachomatis* does induce apoptosis. Cytotoxicity of *C. trachomatis* infected cells has been shown to be caused by a protein coded for by the clostridial toxin B gene present in the Chlamydia genome and LGV-2 serovar seems not to posses this gene (Belland *et al.*, 2001). However not much is known about the rest of the serovars. More work needs to be done on comparing the OG strains with LGV strains, in terms of how they kill cells i.e. type of cell death and whether there is a difference in cytotoxicity levels amongst the strains on endothelial cells. This study aims to identify whether there is any cytotoxic effect and/or cell death in endothelial cells infected with *C. trachomatis* and if there is, which mechanism of cell death occurs and whether there are differences in that respect between LGV and OG strains.

This comparison includes:

i. Production of chemokines; IL-8 and MCP-1 and insoluble intercellular cell adhesion mol-1(ICAM-1) by endothelial cells infected with the two biovars.

ii. Promotion of transendothelial migration of neutrophils and monocytes by the two biovars.

iii. Differences in endothelial cell death and mechanism of death i.e. by apoptosis or necrosis.

iv. Cytotoxic effect during infection

CHAPTER 2: LITERATURE REVIEW

2.1 *Chlamydia trachomatis*

2.1.1 Classification

C. trachomatis belongs to the genus *Chlamydia* which originally had 2 species, *C. psittaci* being the second species. Later a third species *C. pneumoniae* was discovered by Gryston *et al.* in 1989 which was later, together with *C. psittaci* classified in a different genus, *Chlamydophila*. Currently the family *Chlamydiaceae* consists of two genera, *Chlamydia* and *Chlamydophila* and belongs to the order *Chlamydiales,* which falls under the phylum *Chlamydiae* (Everett *et al*., 1999).

2.1.2 Characteristics and Morphology

C. trachomatis has an outer membrane which resembles that of Gram negative bacteria hence Chlamydia are classified as gram negative bacteria. Different from most bacteria, Chlamydia do not have N-acetyl muramic acid in their cell wall. They are aerobic obligate intracellular parasites (Fukuda *et al.*, 2005). These bacteria depend entirely on their eukaryotic host cells to synthesize ATP; hence they cannot survive outside the host cell and can only be propagated in tissue culture systems. Their ribosomes are of prokaryotic origin and they have both DNA and RNA in their nucleus (Barnes, 1989).

2.1.3 Life-Cycle of *Chlamydia trachomatis*

Chlamydia trachomatis has been shown to have a biphasic lifestyle. It infects the host cell as an elementary body which is 0.3µm in diameter. The elementary body (EB) is metabolically inactive and resembles a spore. Infection occurs through parasite specific endocytosis after adhesion of *C. trachomatis* to the target cell. The *C. trachomatis* cell remains engulfed in the phagosome preventing phagolysosomal fusion. Whilst in the host cell, the elementary body transforms after 8-10 hours into a metabolically active form called the reticulate body (RB). This process of transformation from EB to RB has been shown to be characterized by 2 biochemical events. Firstly there is synthesis of chlamydial protein followed by the reduction of the Major Outer Membrane Protein (MOMP) with loosening of its cross-linked disulfide bonds (Moulder, 1991). However more investigations still need to be done to elucidate what really happens during this stage.

The reticulate body starts to divide by binary fission and replicates every 2-3 hours within an inclusion body. This inclusion body is equivalent with the original phago-endosome. On Giemsa stain, the inclusion bodies are vacuolar and granular in appearance. They are single and refract light when viewed under a bright-field microscope. The replication of the RBs within the inclusion body goes on for about 20 hours. Mature RBs then condense and the disulfide bridges of the MOMPs develop allowing the mature RBs to redifferentiate into elementary bodies. One phagosome has been shown to produce about 100-1000 elementary bodies. These elementary bodies then leave the cell by exocytosis (Barnes, 1989; Fukuda *et al.*, 2005).

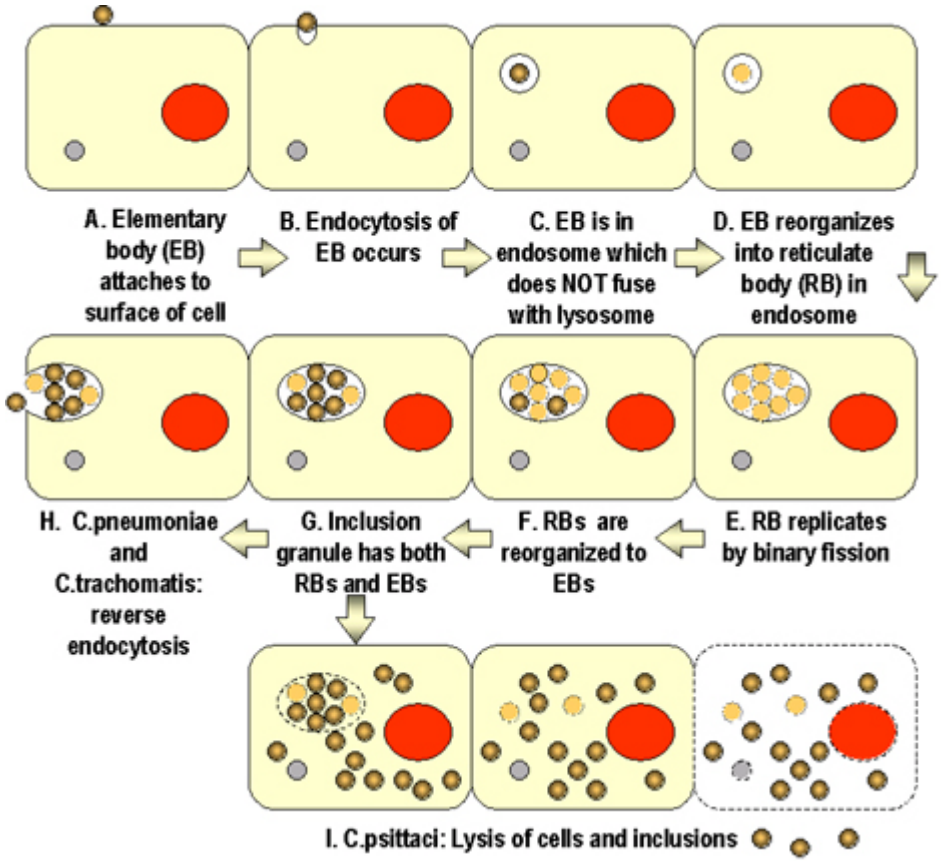


Figure 1: Summary of *Chlamydia trachomatis* lifecycle, from Microbiology and Immunology on-line by Gene Mayer.

2.1.4 Biovars and serovars of *Chlamydia trachomatis*

Chlamydia trachomatis is classified in to two human biological variants according to the characteristics of the disease caused by each group .i.e. Lymphogranuloma Venereum (LGV) and Oculogenital (OG) biovars (Gomes *et al.*, 2004).In the 1960s the biovars were further divided into serotypes/serovars by measuring the level of toxicity of *C. trachomatis* strains on mice after exposure to antibodies. This is known as the "mouse toxicity prevention test" (Barnes, 1989). Wang and Grayston (1974) in further elucidated the serovars serologically by using a microimmunoflouresce test using monoclonal antibodies (MAb) against MOMP. Although Gomes *et al.* (2004)

argues that the phylogenetic data does not entirely offer a definite grouping of the serovars based on their tissue tropism and virulence characteristics, the grouping of serovars into biovars is generally accepted. The OG biovar is made up of serovars A-C and Ba, which cause trachoma, and serovars D-K which cause urogenital infections. The LGV biovar consists of serovars L1, L2, L2a and L3 (Gomes *et al.*, 2004). These serovars have been shown to be more invasive than the OG serovars. Animal models have also demonstrated the LGV biovar to be more virulent than OG biovar (Mabey and Peeling, 2002). Whereas the OG serovars infect the mucosal epithelial surface of the eye and genital tract and are mainly confined to that position, Mabey and Peeling (2002) state that, "LGV seems to primarily infect monocytes and macrophages and can pass through the epithelial layer infecting the regional lymph nodes and may be dispersed throughout the body causing disseminated infection".

2.1.5 Chlamydia infections

2.1.5.1 Lymphogranuloma Venereum

The pathogenesis and manifestation of LGV have shown to vary, but the most distinctive presentation is genital bubonic disease. The classic manifestation is as follows: after 3-30 days of incubation, a small painless lesion which can go unnoticed by the infected individual occurs at the site of infection. This is followed by inflammation of inguinal lymph nodes. Lymphadenopathy of rectum and the anus can also occur. These nodes can develop into painful buboes and ultimately burst and drain.

Involvement of the inguinal lymph nodes is more common in men due to the lymphatic drainage pathway, as for women, their lymphatic drainage of the vagina and cervix is to the retroperitoneal lymphnodes i.e rectal and perianal. Hence women tend to suffer from proctitis. Homosexual men who are receptive during anal sex can also develop proctitis. Patients can also experience headaches, fever and painful muscles. If not treated, hindrance of the lymphatic drainage can lead to elephantiasis in the involved area. Ulcers, stricture and fistulae can occur in the rectal area. (Mabey and Peeling, 2002).

2.1.5.2 Urogenital Infection

Infection with *C. trachomatis* serovar D-K can cause both asymptomatic and symptomatic urogenital infection (Morre' *et al.,* 2000). The common manifestation of this infection in men is urethritis. This is characterized by burning and pain during urination, as well as discharge and inflammation of the urethra. If the anus was the point of contact, it can get inflamed, tender and painful; oro-genital contact can result in a sore throat, .i.e. pharyngitis. The main manifestation in women is cervicits. Lower abdominal pain can occur as a result of pelvic inflammatory disease (PID). PID can lead to ectopic pregnancies (Barnes, 1989; Morre' *et al.,* 2000).

2.1.5.3 Trachoma

Trachoma is a chronic infection of the eye caused by continual infection of the conjuctiva and cornea by *C.trachomtis* serovar A-C. Consequently there is inflammation of the conjunctiva and follicle formation. This in turn leads to scarring

of the conjuctiva which ultimately result in turning in of eyelids. If the cornea also get affected it results in blindness (Barnes, 1989).

2.1.6 Epidemiology of *Chlamydia trachomatis* infections

The epidemiology of LGV is not clearly defined because it is clinically difficult to positively differentiate it from other STDs which cause genital ulcers with formation of a bubo such as chancroid. Diagnosis in the laboratory is more reliable if molecular techniques are applied (Sturm *et al.,* 2005; Alexander *et al.,* 2008). The disease is endemic in parts of Asia, Africa (including Southern Africa), the Caribbean and South America and it is rare in industrialized countries (Mabey and Peeling, 2002). LGV is now the second most common infection (Table 1; Sturm, unpublished).

Table 1. Temporal change in the aetiology of genital ulcers in Durban, South Africa.

Trends in the aetiology of genital ulcer disease in Durban								
	percentage of patients							
	1988 (n=100)	1993 (n=199)	1995 (n=202)	1998 (n=400)	2000 (n=548)	2001 (n=176)	2002 (n=180)	2004 (n=162)
Primary syphilis	42	14	10	43	20	22	9	4
Chancroid	22	53	38	6	8	7	1	1
Genital herpes	10	36	11	40	48	51	52	41
Lymphogranuloma venereum	6		4	3	11	10	14	16
Granuloma inguinale	11		6	4	1.5		0.5	0

Urogenital infections are also spread worldwide (Barnes, 1989) but, like for LGV not much is known about the epidemiology because of difficulties in differentiating between Chlamydia urethritis with other aetiology. *C. trachomatis* is the main cause of urethritis in developed countries while in developing parts of the world other causes play a major role as well (Table 2; (Sturm *et al*. 2004).

Table 2 Aetiology of male urethritis in Durban (n=342)	
	n (%)
Neisseria gonorrhoeae	174 (52)
Chlamydia trachomatis	55 (16)
Trichomonas vaginalis	19 (6)
Mycoplasma genitalium	17 (5)
Ureaplasma urealyticum	121 (36)

The epidemiology of trachoma on the other hand can easily be established. Generally it is a common cause of blindness worldwide, World Health Organization (WHO) states that over 140 million people are infected, and it is estimated that more than 500 million people are at risk of getting the disease. Trachoma is most prevalent in developing countries such as Asia and Africa, and also in Latin America. Approximately 6 million people are blind in the above mentioned geolocations due to trachoma and it is 2 to 3 times more common in women than in men. It is mostly seen in overcrowded and poverty stricken places. Poor hygiene seems to be a major factor facilitating the spread of the disease (WHO, 2010).

2.1.7 Laboratory detection of *Chlamydia trachomatis*

During the 1980s a major breakthrough was made with the development of tissue culture as a means of diagnosing infection with *C. trachomatis*. Culture detection methods are based in detection of viable organisms and these are detected as inclusion bodies by immunofluorescence (Chernesky, 2005). A number of commercial kits to detect chlamydial antigens have been developed. These include Enzyme Immunoassays (EIA) and Direct Immuno-Fluorescence Antibody (DFA) tests (Chernesky *et al.*, 1986; Gift *et al*., 1999). Since tissue culture is too cumbersome for routine use and antigen detection tests lack sensitivity, nowadays these have been

replaced largely by molecular methods. These are also commercially available. However, these tests do not differentiate between biovars.

Serology tests for the detection of antibodies have been used since the 1970s. The method of choice is the microimmunofluorescence test developed by Wang and Grayston in 1974. This test is technically demanding and lacks specificity. Antibody detection tests are only recommended for patients with tubal factor infertility, neonates, and occasionally when there are no bubo aspirates for LGV infections (Chernesky, 2005).

2.1.8 Treatment of Chlamydia infections

Uncomplicated Chlamydia infections which are at an early stage can be treated using antibiotics. The recommended antibiotics are azithromycin and doxycycline (CDC, 2006). However other antibiotics from the macrolide or azolide group and tetracyclines can also be used. (Marrioti, 2004).

2.2 CHEMOKINES AND CELL ADHESION MOLECULES

Chemokines and Cell Adhesion Molecules (CAMs) are protein molecules which play a major role in immunological responses. Chemokines are protein molecules secreted by cells to recruit immune cells. Chemokines can be either be proinflammatory thus involved in recruiting cells to a site of infection or homeostatic in that they control circulation of immune cells in the body to maintain tissue balance and development. Chemokines acquire their name from their chemotaxis nature to neighbouring

responsive cells, hence chemotactic cytokines or chemokines (Zhang *et al.,* 1994; Fernandez and Lolis, 2002). These proteins share common structural characteristics such as their conserved primary structures. They are about 8-10 kilo Daltons in size and have four cysteines in a particular position. Chemokines are grouped into two subfamilies: the C-C proteins family and the C-X-C proteins family and this is based on their structure and genetics (Zhang *et al.,* 1994).

Chemokines and Cell Adhesion Molecules are proteins situated in the surface of the cell. Their functions involve binding to other cells as an immune response and to the extra cellular matrix. They have three domains, namely: intracellular, transmembrane and an extracellular domain. Cell adhesion molecules are grouped into four families, which are cadherins, selectins (these two are also classified as calcium dependent CAMs), intergrins and immunoglobulin superfamily (IgSF). The first two groups are calcium dependent CAMs while the latter two are classified as calcium independent CAMs (Brackenbury *et al.,* 1981).

2.2.1 Interleukin-8

Interleukin-8(IL-8) is produced by numerous cell types such as macrophages, epithelial cells and endothelial cells. Utgaard *et al.* (1998) has shown that unstimulated microvascular endothelial cells such as human intestinal microvascular endothelial cells (HMIEC) and polyp microvascular endothelial cells (PMEC) contain lL-8 in intracellular vesicles known as a Weibel Palade bodies. However these IL-8 containing granules are not present in unstimulated HUVEC. In this endothelial cell line IL-8 is only expressed on stimulation.

Based on its structural characteristics IL-8 belongs to the C-X-C subfamily of chemokines. This group unlike the C-C subfamily is known to have another amino acid between two cysteine residues situated near the N-terminus (Skelton *et al.*, 1999). IL-8 has the four cysteines typical of any chemokine structure. These cysteines are joined by intramolecular disulfides bonds, with the first cysteine joined to the third one and the second joined to the fourth cysteines. The first two cysteines as already mentioned are situated near the N-terminus, the third is found in the centre and the last one in the C-terminus of the molecule (Fernandez *et al.*, 2002).

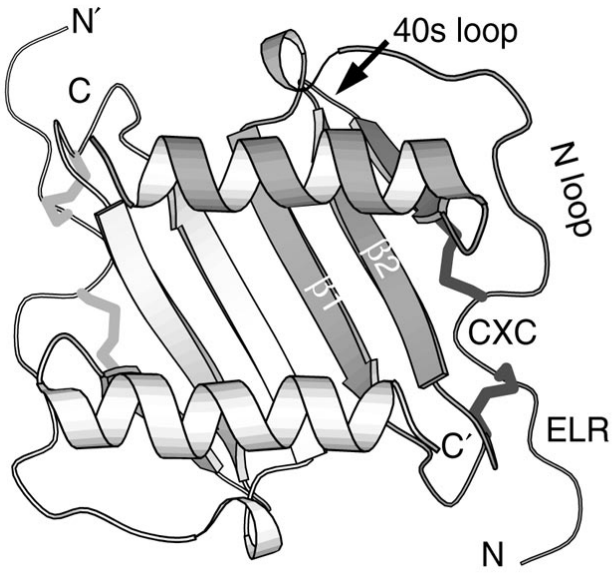


Figure 2: Overview of the IL-8 dimer structure adapted from Skelton *et al.* (1999). Showing dimerization of the C-X-C through the N-terminal β-strand resulting in a six-stranded β-sheet forming compact nearly spherical dimers. Also indicated is the Glu-Leu-Arg (ELR) amino acid sequence near the N-terminus and residues in the N loop.

Interaction of the loops of the amino acids and cysteines form a tertiary structure with 3β sheets anti-parallel to each other and an α- helix at the C-terminus. Dimerization of the C-X-C is normally through the N-terminal β-strand resulting in a six-stranded

β-sheet (Lowman *et al.,* 1996), which form compact nearly spherical dimers (Zhang *et al.,* 1996; Skelton *et al.,* 1999). C-X-C chemokines are further divided into 2 categories, ELR positive and ELR negative. The ELR positive category which IL-8 belongs to, have a Glu-Leu-Arg (in short ELR) amino acid sequence near the N-terminus. The ELR and residues in the N loop have been shown to be important in binding of the chemokine to its receptors (Lowman *et al.,* 1996).

Interleukin-8 is involved in innate immunity. It acts as a chemoattractant. It primarily recruits neutrophils to the site of inflammation. This is why it is also referred to as neutrophil chemotactic factor. IL8 is produced by infected cells, for instance endothelial cells and released into the surrounding tissue. Neutrophils migrate in the direction of the chemokine producing cells. In this process IL8 binds to the IL8 receptors receptors in the membrane of the neutrophil. This is followed by removal of the chemokine to maintain a concentration gradient in the direction of the producing cell layer. MCP-1 functions in a similar way, macrophages instead of neutrophils (Utgaard *et al.,* 1998).

2.2.2 Monocyte Chemotactic Protein-1

Monocyte Chemotactic Protein-1(MCP-1) is produced by a number of cells following stimulation. These cells are endothelium, epithelium, smooth muscle cells, fibroblasts and mononuclear leukocytes. (Hasegawa *et al.,* 1999).

MCP-1 belongs to the C-C subfamily of chemokines, which unlike the C-X-C do not have an amino acid between the two cysteines near the N-terminus, hence no ‘X’

between the ‘CC’. Like with all chemokines the dimerization of MCP-1 results into 3 anti-parallel β-sheets (β1, β2, and β3) from the N-terminus to C-terminus, however unlike the C-X-C subfamily in which the dimer is found mainly between β1 of two subunits, the dimmers of C-C chemokines occur near the N-terminus along a β0 sheet. This results in long cylindrical dimers typical of MCP-1 (Figure 3, Zhang *et al.*, 1996). Mutagenesis studies by Zhang *et al.* (1996) have shown that the N terminus of MCP-1, particularly Asp-3, plays an important role in chemo attraction.

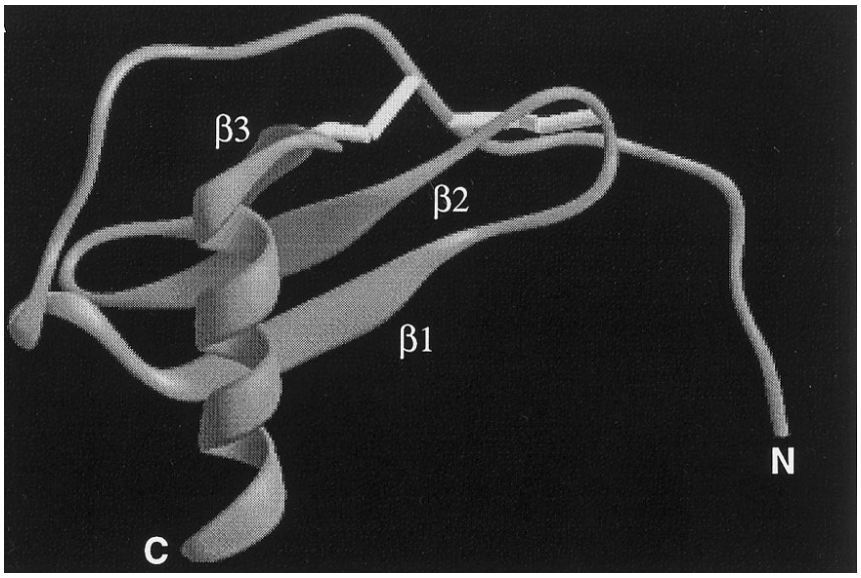


Figure 3: Structure of MCP-1β monomer adapted from Zhang *et al*. (1996) showing three β-sheets (β1, β2, and β3) and the amino (N) and carboxy(C) termini. The angular cylinders indicate the disulfides bonds.

Like all chemokines, it acts as a chemo attractant, recruiting monocytes to the site of infection where they mature into macrophages (Skelton *et al.*, 1999)

2.2.3 Intercellular Cell Adhesion Molecule-1

Intercellular cell adhesion molecule-1 (ICAM-1) is produced by activated endothelial cells. The human gene which encodes for ICAM-1 is located in chromosome 19 and

its expression can be induced by a range of mediators including infectious agents (Rahman and Fazal, 2009)

Intercellular cell adhesion molecule-1 is a cell surface glycoprotein with a molecular weight ranging from 76 to 114 kDa and consists of 505 amino acids. This glycoprotein belongs to the immunoglobulin super family. It has 5 extracellular Ig-like domains (D1-D5), a hydrophobic transmembrane domain, and a short cytoplasmic domain of 28 amino acids (Staunton *et al.*,1988; Springer, 1990;). The D1 domain acts as a binding site for Leukocyte Function-associated Antigen (LFA-1) whereas D3 acts as the binding site for Macrophage-1 antigen (Mac-1). (Fig. 4) (Bella *et al.*, 1998).

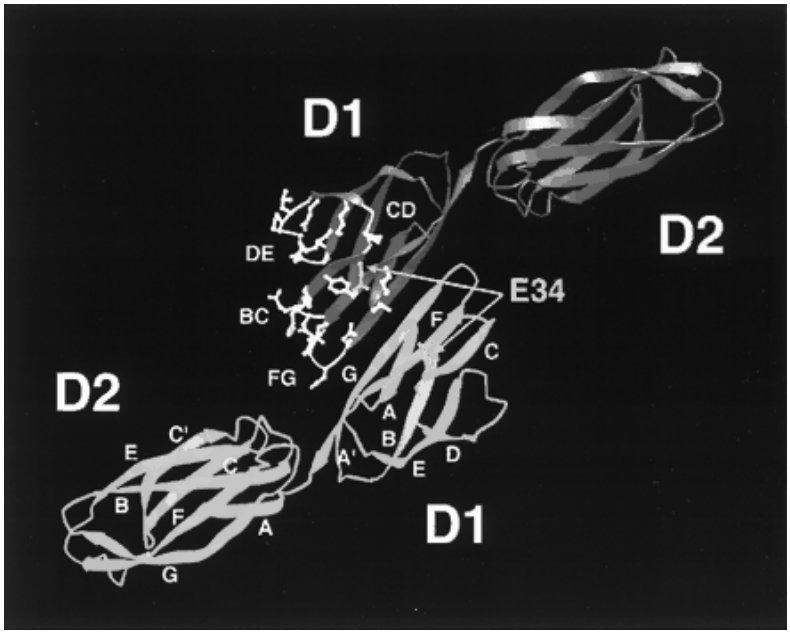


Figure 4: The structure of the ICAM-1 dimer adapted from Bella *et al.* (1998) showing the crystallographic dimer with D1 and D2 domains and antiparallel ß-sheets between the G strands.

Binding of ICAM-1 to ß-intergrins allows adhesion of the recruited neutrophils, monocytes and lymphocytes to the endothelium resulting in transendothelial migration also known as diapedesis. The first role involves the extracellular domain which has been shown to facilitate firm adhesion of leukocytes to the endothelium (Lyck *et al.*, 2003; Engelhardt and Wolburg, 2004). The second role involves the cytoplasmic domain which has been indicated by mutagenesis studies to play an important role in promoting transendothelial migration of leukocytes (Greenwood *et al.*, 2003; Lyck *et al*., 2003).

2.3 ENDOTHELIAL CELLS

Endothelial cells line the lumina of blood and lymphatic vessels, forming the endothelium (Nisato *et al.*, 2004). The cells are flat with a diameter of 10-20 µm and approximately 1-2 µm thick giving the endothelium a pavement –like appearance (Fig. 5).

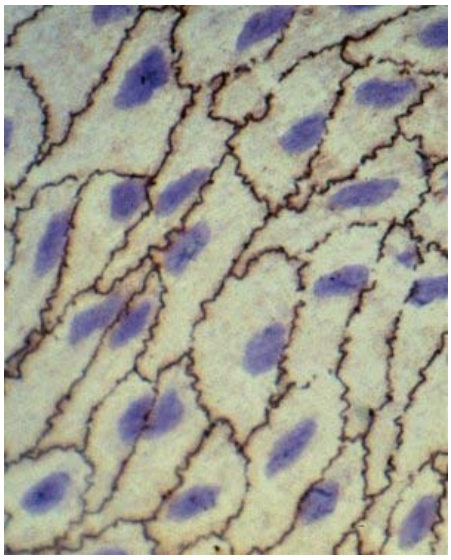


Figure 5: showing endothelial cells with the blue nucleus and the silver nitrate stained black intercellular junctions. Original magnification 400X. (From: School of Anatomy and Human Biology, University of Western Australia).

Endothelial cells are involved in a range of vital physiological processes. They have been shown to secrete mediators which may induce biological reactions by an assortment of signal-transduction mechanisms. These mediators such as chemokines and cell adhesion molecules act as attractants and binding sites for various cellular immune components facilitating diapedesis. Endothelial cells also act as discriminatory filters by controlling the passing of soluble molecules. They are also involved in angiogenesis, which is the formation of new blood vessels (Farjado, 1989).

2.4 EXTRAVASATION/ TRANSENDOTHELIAL MIGRATION

Infection or injury to the body results in a cascade of events known as the inflammatory response. This response provides a mechanism to rid the host of infection and to repair the damage (Fig. 6). A major component of the inflammatory reaction is for the leukocytes to migrate from the blood through the endothelium to the infected tissue, a process known as extravasation. This transendothelial migration (TEM) of leukocytes is a complex, well regulated multistep process which involves a number of signaling molecules. The first step in TEM encompasses rolling. In the absence of any injury or infection the endothelial layer and the intra-vascular leukocytes are in a resting state in which there is no interaction. Upon stimulation the endothelial cell start secreting chemokines which recruit the leukocytes and the expression of selection ligands, which in turn capture the leukocytes from the bloodstream by “tethering”. The chemokines are leukocyte receptor specific (Krüll *et al.*, 1999; Wang. and Grayston, 2002; Rahman and Fazal, 2009).

Some chemokines are broad acting whereas others are leukocyte type specific. For instance, Platelet Activating Factor (PAF) has been shown to stimulate numerous types of leukocytes (Zimmerman, 1992; Simon *et al.*, 1994) whereas IL-8 and MCP-1 are specific for recruiting neutrophils and macrophages respectively (Utgaard *et al.*, 1998). Broad acting transmembrane protein P-selectin has been shown to be the most rapid selectin in recruiting neutrophils and is able to maintain the initial rolling of neutrophils. Rolling is temporary and is stopped when firm adhesion occurs through the binding of the integrin which necessitates $G\alpha_1$-linked receptors. PAF and IL-8 have been shown to activate this $G\alpha_1$ pathway. Utgaard *et al.* (1998) further argues that IL-8 has the advantage over PAF by being able to support the subsequent firm binding of neutrophils by $G\alpha_1$ receptors, and its rapid release. It is restricted to neutrophils hence more specific. Even though not much work has been done in this regard for MCP-1 as compared to the IL-8 chemokine, MCP-1 is also leukocyte-type specific and hence it is safe to say that it posses the same advantages that IL-8 has compared to broad acting chemoattractants.

The 2^{nd} step is the firm adhesion, arrest or tight binding of leukocytes to the endothelium. In this stage there is up-regulation of expression of ICAM-1 receptors on the activated endothelial cell surface. Molecules that bind to the endothelial receptors are activated on the leukocytes like β_2 integrins, LFA-1, $\alpha_1\beta_2$, CD11a (CD18) and Mac-1($\alpha M\beta_2$, CD 11b/ CD18). The ICAM-1 interacts with receptors on the leukocytes facilitating the arrest or tight adhesion. This in turn makes it possible for the leukocytes to pass across the endothelium through a process known as

diapedesis which is followed by migration to the affected tissue (Krüll *et al.*, 1999; Rahman and Fazal, 2009).

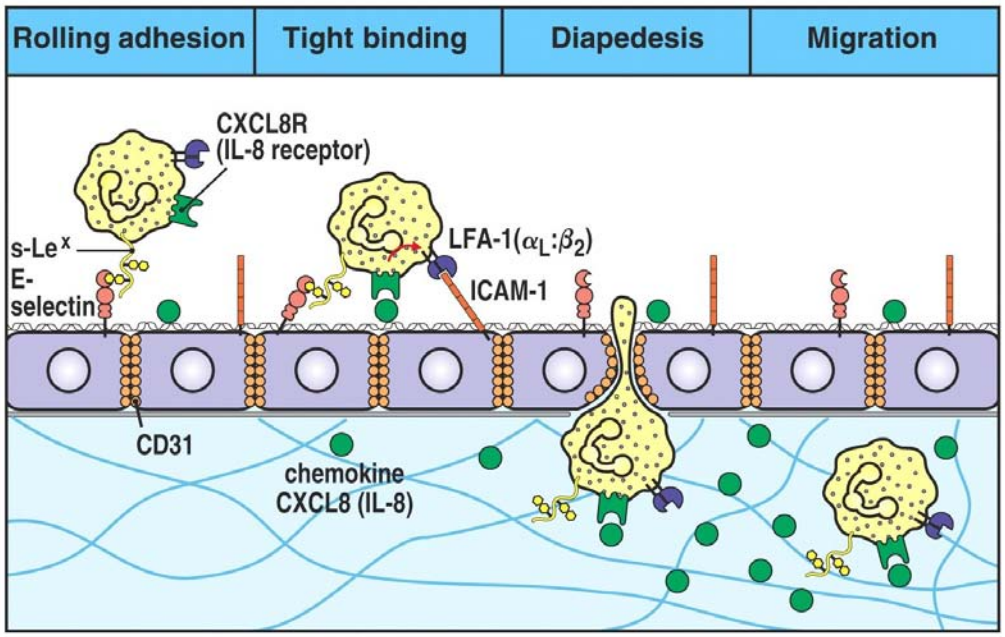


Figure 2-44 part 3 of 3 Immunobiology, 6/e. (© Garland Science 2005)

Figure 6: A summary of the steps that occur during transendothelial migration, adapted from Garland Science 2005.

There has been many debates on which route is taken by the leukocytes during this transmigration, is it transcellular or paracellular? In ex-vivo experiments, the path the leukocytes take seem to be dependent on the type of stimuli, activation state, origin of endothelial cells (HUVEC, HMIEC, PMEC) and type of leukocytes, however further studies still need to be done to elucidate and validate the determinants for each route (Rahman and Fazal, 2009).

2.5 INTERACTION OF CHLAMYDIA WITH ENDOTHELIAL CELLS

2.5.1 Production of Chemokines and Adhesion molecules

Chlamydia spp has been shown to induce the production of chemokines and expression of adhesion molecules on the infected host cell. These in turn play a role in recruiting leukocytes to the site of infection. This is part of the inflammatory response which is the hallmark of Chlamydia infection. Chlamydia is one of the pathogens which cause destructive delayed chronic inflammatory response (Stephens, 2003). Chlamydia infects non immune cells and elicits the production of proinflammatory molecules such as IL-6, IL-1α, IL-11, IL-8 and MCP-1. This results in an acute inflammatory response followed by a chronic phase which is characterized by the build up of a mononuclear infiltrate including macrophages and lymphocytes. These inflammatory responses consequently lead to cell proliferation and damage to the tissue (Stephens, 2003; Ward, 1999).

Several studies have been done on interaction of *Chlamydia* spp with host cells. Krüll *et al*. (2004) reported that *C. trachomatis* serovar K was able to infect HUVEC. After an exposure period of 24 hours, the bacteria did infect the cells, but this did not stimulate any production of IL-8 or cell surface ICAM-1. These molecules were however induced when HUVEC was infected with *C. pneumoniae* strain TW 183. The authors also referred to their preliminary results on *C. trachomatis* serovar L2 and E stating that even though both these serovars successfully infected HUVEC, there was no production of IL-8 and MCP-1. Therefore, *C. pneumoniae* was able to induce

early and rapid signal transduction but the different serovars of *C. trachomatis* did not.

Fukuda *et al*. (2005) showed that both *C. trachomatis* LGV-2 and E did induce production of IL-8 but by peripheral blood mononuclear cells (PBMC) and epithelial cells. This raises the question whether this feature is unique for epithelial cells and PBMCs only. Similar results were reported by Molestina *et al.* in 1998 who reported that *C. pneumoniae* induced the expression of not only IL-8, but MCP-1 and sICAM-1 by HUVEC after 24 hours. Fukuda's data confirm observations on the expression of cell bound E-selectin, ICAM-1 and VCAM-1 by *C. pneumoniae* infected HUVEC made formerly by Krüll *et al*. (1999).

2.5.2 Transendothelial migration of neutrophils and monocytes

As already mentioned chemokine and cell adhesion expression leads to recruitment of immune cells to the site of infection which in turn result in transendothelial migration. Few studies have been done on transendothelial migration of leukocytes during Chlamydial infection. A study done by Molestina and colleagues in 1999 even though the main focus was on *C. pneumoniae* also looked at *C. trachomatis* infection. The authors reported that *C. trachomatis* L2 demonstrated a minor raise in neutrophil transendothelial migration and a significant transendothelial migration of monocytes compared to mock-infected cells. This was regardless of lack of IL-8 induction or MCP-1 induction. They attributed this to the possibility that a different chemotactic factor was responsible for this monocyte migration.

The oculogenital serovars A and E failed to induce MCP-1 and IL-8 stimulation in HUVEC. *C. pneumoniae* however promoted up regulation of IL-8 and MCP-1 and also promoted transendothelial migration of both neutrophils and monocytes. They then concluded that up regulation of MCP-1 and IL-8 by endothelial cells was unique to *C. pneumoniae*. However, *C. trachomatis* up regulated IL-8 and MCP-1 in monocytes and epithelial cells. Hence the authors concluded that stimulation of chemokine production is cell type specific.

2.6 CELL DEATH

2.6.1 Overview

Pathogen induced cell death was first described by Hirano and Ruebner in 1966. As time went on it was recognized that this occurs in a regulated and controlled manner and plays a major role in the pathogenesis of disease. In order to survive inside the host some micro organisms may kill the cell responsible for immunity as a form of defence. Some micro organisms especially those that live intracellularly, don't instantly kill the host cell because they need it for replication and growth. However lysis will occur when the multiplied organism is ready to infect other host cells. Cell death induced by pathogens happens by different mechanisms as descibed in the paragraphs below.

2.6.2. Apoptosis

Apoptosis is a Greek word which means “falling”. Kerr *et al.* (1972) first described apoptosis based on morphological changes. Those included cytoplasmic and nucleus condensation, followed by disintegration of the cell into membrane-bound fragments known as apoptotic bodies. The plasma membrane remains intact. Apoptosis does not cause an inflammatory response because the apoptotic bodies are taken up by neighbouring cells in phagosomes. Apoptosis is a controlled mechanism of cell deletion, hence termed “programmed cell death”. It is used to remove dysfunctional cells.

A study done on abnormal cell mutants by Hedgecock and colleagues in 1983 revealed that there were gene products concerned with cell death during the development of an embryo. These products belonged to a family of proteases known as caspases. However, not all caspases are involved in apoptosis. The caspases involved in apoptosis include caspase 2, 3, 6, 7, 8, 9 and 10 (Creagh *et al.*, 2003). These caspases are further grouped into two subgroups on the basis of their role in activating cell death and their structure. The first group comprises of initiator caspases, which are 2, 8, 9, and 10. As the name implies they are involved in initiating the apoptotic cascade by mediating dimerization which is needed for the caspase activation. The second group consists of caspase 3, 6 and 7 which are involved in cleaving and disassembling of the cell. They are activated through proteolysis (Boatright and Salvesen, 2003).

Some pathogens have been shown to be able to activate the caspase cascade hence inducing cell death. For instance *C.trachomatis* has been shown to manufacture a protein known as Chlamydia protein which activates apoptotic caspases by interacting with the Tumor Necrosis Factor family receptor death domains (Stenner-Liewen *et al.,* 2002).

It follows from the above that apoptosis cannot only be studied based on morphological changes but also based on the biochemical pathways involved. Therefore apoptosis is defined as caspase-mediated cell death which leads to the already mentioned morphological changes and elimination of the resulting apoptotic bodies by phagocytosis (Samali *et al.,* 1999; Blagonsklonny, 2000).

2.6.3 Necrosis

The term necrosis is used to define any death of cells which is not apoptotic. Necrotic cell death differs from apoptosis in that it does not activate any intra-cellular biochemical process that leads to death of the cell (Levin, 1998; Levin *et al.,* 1999). Necrotic cell death is accompanied by cell lysis. Apoptotic cells which are not eliminated by phagocytosis can disintegrate and proceed to a stage known as apoptotic necrosis. Majno and Joris (1995) argues that it is possible for cells close to apoptotic ones to undergo necrosis even though apoptosis is regarded as a process whereby disposing of the dead cells causes no disruption.

2.6.4 Oncosis

Even though apoptosis and necrosis are the major mechanisms of cell death, more types of cell death have been proposed. One of them is oncosis. This term is used to describe the prelethal process which is characterised by organelle and cellular swelling. The membrane permeability also increases and breaks down. Even though the nucleus swells it does not fragment. Oncosis results in ATP depletion due to processes which cause consumption of energy in an uncontrollable manner. Some of this energy depletion has been shown to be caused by biochemical reactions catalysed by enzymes. For instance in an attempt to repair massive DNA damage, poly 2.4-di-amino-6-pyrimidine (DAP)-ribose polymerase (PARP) increases its intake of its substrate nicotinamide adenine dinucleotide (NAD) which it uses to repair DNA, This means NAD has to be resynthesised which can result in depletion of ATP and subsequent death of the cell due to lack of energy (Walisser *et al.,* 1999). Induction of oncosis by pathogens has been demonstrated on MA 104 cells infected with Rotavirus and *Pseudomonas aeruginosa* infected macrophages and neutrophils (Dacheux *et al.*, 2000).

2.6.5 Autophagy

Another proposed type of cell death is autophagy. This refers to the degradation of cellular debris within the dying cell in vacuoles called autophagic vacuoles. This is also known as Type II cell death. The morphological changes involved are vacuolisation, cytoplasmic contents degradation and minor condensation of the chromatin. Autophagic cells can also be phagocysed. Autophagy does not cause any

inflammatory reaction since the components are degraded in autophagic vacuoles. (Clarke, 1990; Bursch, 2001).

2.6.6 Pyroptosis

The last type of cell death is pyroptosis. This form of death involves Caspase 1 activity and results in the breakdown of the cytoplasmic membrane. Hence it leads to a proinflammatory reaction. Caspase-1 activates the inflammatory cytokines IL-1β and IL-18 proforms. There are some pathogens which have been shown to cause cell death by the pyroptosis pathway. Some studies have shown that salmonella and shigella cause pyroptosis in macrophages (Zychlinsky *et al.*, 1994; Hersh *et al.*, 1999; Obregon *et al.*, 2003).

Fink and Cookson (2005) suggest that that there are other types of cell death that there are still undiscovered or uncharacterised. They further elaborate that death of a cell may involve numerous pathways at the same time and communication between the pathways can allow a controlled form of death. They postulate that no distinct cell death programme is responsible for the death of a cell, but that there is an overlap of several cell death types. This is further supported by former studies by Ankarcrona *et al.* (1995) and Kostin *et al.* (2003).

2.6.7 Measurement of Cell death

Although the understanding of mechanisms of cell death is improving, methods to study this are complicated. Fink and Cookson. (2005) argue that most of the methods

to establish and quantitate apoptosis are inadequate to prove that no other cell death pathways occur at the same time. For instance different stains and fluorescent dyes are used to identify condensed chromatin but oncotic cells also show condensed chromatin. Visualising of DNA fragmentation by gel electrophoresis is also used since it was formerly described as a trademark of apoptosis. However, necrotic cells also have been shown to have nuclear fragmentation (Collins *et al.*, 1992) and TUNEL positive nuclei (Grasl-Kraupp *et al.*, 1995; de Torres *et al.*, 1997). Measurement of lactate dehydrogenase is used to identify necrosis since as the cell looses its integrity it releases cytoplasmic enzymes. Even though it shows that the cell membrane is destroyed it doesn't provide information on which pathway led to that. The annexin V test which measures phosphatidylserine (PS), has been shown not to be able to discriminate between oncotic, necrotic and apoptotic cells (Lecoeur *et al.*, 2001).

Caspase activity measurement is considered to be more specific than the other methods. This test is based on conjugation and proteolysis of tetrapeptide substrates with caspase cleavage sites. The products formed are detected by means of fluorometric or colorimetric assays. The problem with this test is that it measures the enzyme activity in a population of cells and not in a single cell. Conjugating caspase inhibitors with peptide markers which mimic the caspase cleavage sites can lead to non-specific binding due to overlapping of caspase activity (Thornberry *et al.*, 1997; Villa *et al.*, 1997).

With all these test limitations, cell death measurements are limited to measuring cell membrane integrity and the classic markers of apopotosis. So far, there are no established techniques to identify other types of cell death.

2.6.8 Chlamydia induced Cell death

Since *Chlamydia trachomatis* is an obligate intracellular organism, it needs its host cell for survival. Hence, it has developed strategies to keep the host cell viable following infection. However the pathogen needs to exit the infected cell, and escape the immune response of the host cell. This can be achieved by inducing apoptosis during which cell contents, including the intracellular pathogen, is packed in membrane bound compartments. There are contradicting reports with some research saying Chlamydia is anti-apoptotic and others saying Chlamydia infection does induce apoptosis.

Samuel and Stephens (2001) showed that there was phosphatidylserine (PS) externalisation on the surface of human endothelial, epithelial, monocytic and granulocytic cells infected with *C. trachomatis* (L2) and *C. pneumoniae*. PS externalisation is an indication that the cell is at an early stage of apoptosis. The PS externalisation was immediate and was not affected by a broad spectrum of caspase inhibitors. The authors argue that maybe the caspase cascade is either not needed or bypassed. They therefore concluded that the involvement of caspases to the chlamydia induced apoptotic process is minor. This is consistent with the findings of Ojcius *et al*. (1998). *C. pneumoniae* has also been shown to induce apoptosis in a caspase

independent manner in coronary artery endothelial cells (Schoier *et al.*, 2005). It was also transient .i.e. withdrawal of the stimulus resulted in loss of PS externalisation.

Ojcius did further research in 1999 with Jean-Luc as the first author. They reported that *C. trachomatis* infection of HeLa cells leads to apoptosis. They then proceeded to do *in vitro* studies in mice and they found that the infected mice had a significant amount of apoptotic cells in the uterus and oviducts after 2 to 7 days of infection. However even though they observed apoptotic cells in the endocervix the number was not significant and they argued that it might be due to shedding of the dead cells because large clumps of infected cells were observed there. Their observations were supported by Gibellini *et al*. (1998). They reported that apoptosis was evident on LLC-MK2 cells infected with *C. trachomatis* LGV-2 serotype or with *C. psittasi.*

C. trachomatis has also been shown to have anti-apoptotic properties. There are several mechanisms involved in this anti-apoptotic activity. These include, cleavage of polyADP ribose and inhibition of the activation of caspase 3 in cells infected with Chlamydia (Fan et *al.,* 1998). Blockage of mitochondrial cytochrome C release has also been demonstrated in chlamydia infected cells. It has also been shown that Chlamydia infection of cells leads to degradation of BH3-only proteins, which are *bcl*-2-interacting killer (Bik) protein, p53 upregulated modulator of apoptosis (Puma) and bisindolylmaleimide (Bim). These proteins belong to the B-cell lymphocytic-leukaemia proto-oncogene-2 (*bcl*-2) family and are required for activation of the cytochrome c release mechanism. Their degradation results in loss of pro-apoptotic activity and hence the infected cell is protected against apoptosis (Fischer *et al.*, 2004; Dong *et al.*, 2005; Ying *et al.*, 2005). Dean *et al.,* 2001 also showed that Hela 229

cells infected with *C. trachomatis* resisted apoptotic stimuli in acute and persistent infections. The infected cells were stimulated with etoposide and staurosporine. They observed no DNA ladder and the cytochrome c remained intact in the mitochondria whereas the reverse was true for uninfected cells.

Cytotoxicity by *C. trachomatis* has been described as early as 1944 when Rake and Jones described this in macrophages. In 2001, Belland and colleagues further classified the toxins involved and identified a clostridial toxin B gene encoded in the Chlamydia genome as the one responsible for cytotoxicity. This study was done on epithelial cells. They used strains, D, L2 and MoPn. MoPn was found to be more toxic than serovar D, whereas L2 did not show any cytotoxic effect. This corresponded with the absence in L2 of open reading frames (ORFs) (TC0437-0439) which are responsible for cytotoxity. They reason that lack of cytotoxicy of L2 on epithelial cells allows it to infect monocytes and disseminate to the lymph nodes.

CHAPTER 3: MATERIALS AND METHODS

3.1. CELL CULTURES AND PROPAGATION OF ISOLATES

Chlamydia trachomatis serovars L1 (ATCC® VR-901B), L2 (ATCC® VR-902B), L3 (ATCC® VR-903) (Lymphogranuloma strains) and E (Ocular Genital strain) were used in this study. The LGV strains were kindly donated by Frans Radebe of the National Institute of Communicable Diseases and the serovar E was isolated by Maleka and colleagues (1996) from a male patient presenting with urethritis at the Prince Cyril Zulu Communicable Diseases Clinic in Durban. Typing of this strain was done using the monoclonal antibody microimmunofluoresence test by Dr Lampe at the Department of Medicine in the University of Washington, Seattle. The strains were kept as frozen stocks in the department of Medical Microbiology. McCoy cells were used for propagation of isolates and Human Umbilical Vein Endothelial cells (HUVEC) were used for experimental purposes. McCoy cells (American Type Culture Collection, number CRL-1696) were from frozen stocks whereas pooled cryopreserved HUVEC, in EGM^{TM} -2 from Clonetics®.

3.1.1 McCoy Cells

3.1.1.1. Reconstitution

A vial containing McCoy cells was removed from the -70ºC freezer and allowed to thaw. The vial was wiped with 70% ethanol and placed in a class II biological safety cabinet. The contents of the flask were diluted 1:10 in growth medium. This was done by adding 20ml of Eagle's minimum essential medium (EMEM) manufactured by

Bio-Whittaker^{TM}, Walkersville, MD,USA with L-glutamine (2mM) (Bio-Whittaker^{TM}, Walkersville USA) to a 75cm^2 tissue culture flask. The EMEM was then supplemented with 2ml of heat inactivated foetal bovine serum (FBS). McCoy cells from the vial were then added to the flask. The flask was placed at 37ºC in a CO_2 incubator with 5% CO_2 in air. After 24 hours, the cells were checked for confluency by observing them under an inverted microscope at 200X magnification. If confluency was observed, the cells were trypsinised (3.1.1.2) and if not the medium was changed and the cultures were re-incubated until they reached the desired confluency.

3.1.1.2 Trypsinization

When 90-100 % confluency was reached, the medium was removed and the cell monolayer washed thrice with phosphate buffered saline (PBS, pH 7.4, Oxoid) without calcium and magnesium. The PBS was decanted and replaced with 1 ml of warmed trypsin-EDTA solution (0.05 % trypsin - 0.02 % versene BioWhittaker^{TM}). The flask was swirled gently in order to spread the solution evenly over the cell monolayer. Excess trypsin-EDTA solution was decanted leaving a small amount to cover the surface of the flask. The flask was incubated at 37 ºC for 30-60 seconds and tapped on the palm of the hand to facilitate easy detachment. Immediately after detachment, cells were resuspended in 1 ml of FBS to stop the action of the trypsin. The cell suspension was transferred to a 100 ml bottle containing a 60ml of EMEM with 10 % FCS. Twenty millilitres of the contents of the bottle was aliquoted into each flask. The flasks were labelled with the passage number and incubated at 37 °C with 5 % CO_2 in air.

3.1.1.3 Cell count

Cells were trypsinised as described in 3.1.1.2. A viable cell count was then carried out using the trypan blue dye exclusion assay. This assay is based on the principle that viable cells actively remove the dye from the cell whereas dead cells cannot do that. Thus, viable cells remain colourless and hence viable cells can be differentiated from dead cells under the microscope. Twenty microlitres of cell suspension and 20 µl of 0.4 % trypan blue (sigma®) was mixed in a microcentrifuge tube. If cells were too concentrated they were diluted with PBS prior to the addition of trypan blue solution and the volume of PBS used was noted and included in the dilution factor. A glass coverslip was placed on a haemocytometer. The cell suspension mixed with trypan blue dye was pipetted under the coverslip on one half of the haemocytometer; this was done carefully to make sure that there was no overflow and no air bubbles. The number of cells in the four outer grid boxes of the haemocytometer was counted using a bright field microscope. The following formula was used to determine the concentration of cells per volume unit:

$$\text{Concentration of cells (cells/ml)} = \frac{n}{\text{No of grid boxes}} \times 10^4 \times \text{dilution factor}$$

Where n = total number of cells counted.

3.1.1.4 Propagation of McCoy cells

To prepare cell culture stock, the cells were trypsinised and stored in McCoy cell freezing fluid prepared as described in appendix A. The trypsinised cell suspension was transferred to a 15 ml centrifuge tube. The total volume of cells was noted and

the same volume of freezing fluid was slowly added, drop by drop to the cells, with swirling between the addition of each drop. Aliquots of 1 ml were pipetted into cryovials. The cryovials were labelled with the name of the cell line, date and passage number. The cryovials sealed with parafilm and placed in polystyrene racks to allow slow freezing of the cells and stored in a -70ºC freezer for later use.

3.1.2 Chlamydial serovars

3.1.2.1 Inoculation

Chlamydia trachomatis serovars L1, L2, L3 and E were used in this study. McCoy cells were seeded into trak vials with coverslips at a concentration of 8 x 10^4 cells per vial. Before inoculating the cells, one track vial was examined using an inverted bright field microscope to check whether the cells were 70-80 % confluent. If so, media was aspirated from the remaining vials and replaced with 500 µl chlamydia growth medium (see Appendix A). Nine hundred microlitres of sucrose-phosphate glutamic acid (SPG) buffer (Appendix A) was added to a microcentifuge tube and 100 µl of suspension containing the serovar of interest was added. One hundred microlitres of this mix was then added to the trak vial. One hundred microlitres of SPG buffer was added to the negative control vial. To improve contact between chlamydia and cells, the vials were centrifuged at 1200 x g for 1 hr. This was followed by incubation at 37°C in 5% CO_2 in air for 1 hour. The growth medium was aspirated from each trak vial and the monolayer rinsed once with PBS. This was replaced with 1 ml of chlamydia growth medium. The infected cells were incubated for 48 hours at 37°C in 5% CO_2 in air.

3.1.2.2 Harvesting of chlamydia

The MicroTrak® *C. trachomatis* Culture Confirmation Test Kit (Trinity Biotech) was used to confirm that the organism had infected and grown in the host cells. This was done according to the manufacturer's instructions. Two of the vials were selected for staining, the negative control and one inoculated vial. The media was discarded from the 2 vials, 1 ml of 95% ethanol was added and the vials were incubated at room temperature on the working surface for 5-10 minutes. The ethanol was aspirated and the coverslips were removed and put into wells in a 24-well tissue culture plate. Five hundred microlitres of PBS was added to each vial. Distilled water was pipetted into the remaining empty wells to create a humidified environment. PBS was discarded leaving a small amount at the bottom. Thirty microlitres of the conjugated anti-MOMP antibodies was added to each well. A wet paper towel was then placed on top of the wells and the plate was closed with a lid. The plate was incubated for 30 minutes at room temperature and agitated by hand every 10 minutes. The fluid was removed and the coverslips were washed with distilled water. The coverslips were removed and blotted dry on a paper towel. Coverslips were mounted with the cell containing side down on a glass slide using a drop of Distyrene Plasticizer Xylene (DPX) - mountant (Sigma®). The slide was allowed to dry and then viewed under a fluorescent microscope (Olympus®) with a filter system for fluorescein isothiocynate (FITC) (λ =520nm) at magnification 100-400x (dry objective,). Green fluorescent chlamydial inclusions confirmed the presence of chlamydia and indicated that the organisms could be harvested. This is because the staining solution contains FITC conjugated to a monoclonal antibody directed against the major outer membrane

protein (MOMP) of *C. trachomatis*. This antibody binds to *C. trachomatis* resulting in fluorescence.

Chlamydia was harvested as follows. Media was aspirated from trak vials and replaced with 600 µl of SPG containing 10% FCS. Four glass beads were then added to each trak vial. The contents of the trak vials were pulse vortexed for 30 seconds. Chlamydia was then aspirated and pooled in a 10 ml tube. One hundred and fifty microlitre aliquots of chlamydial suspension were then transferred into microcentrifuge tubes. The tubes were labelled and stored at -70°C to be used for inoculation of endothelial cells.

3.1.2.3 Determination of inclusion body concentration

For determination of inclusion body concentration, McCoy cells were grown in trak vials and inoculated with 10-fold serial dilutions of each isolate (L1, L2, L3 and E) from the stored stocks (3.1.2.2). Dilutions ranged from 10^{-1} to 10^{-4} . Each dilution was inoculated in triplicate. After 48 hours, monolayers were stained by using the *C. trachomatis* culture confirmation kit as described above. The slides were then viewed under the fluorescent microscope. A dilution of each serovar in which the number of inclusions could be counted without difficulty was chosen. Counts of inclusions were carried out at 200X magnification. Twenty fields of view were counted for each triplicate. The results were then averaged and the concentration determined using the following formula:

$$\text{Concentration (IFU/ml)} = n \times \frac{1000\mu l}{100\mu l} \times \text{conversion factor} \times \text{dilution factor}$$

n = average no. of inclusion bodies

See appendix B for a list of conversion factors.

3.1.3 Endothelial Cell cultures

3.1.3.1 Propagation

HUVEC was cultured in 25 cm^2 culture flasks and maintained in endothelial growth medium-2 (EGM-2) (Clonetics®). The EGM-2 medium was aseptically reconstituted by adding the entire contents of each supplement vial to 500 ml of the medium. The supplements added were 2 ml FBS, 0.5 ml gentamicin sulphate, amphotericin-B (GA-1000), 0.5 ml ascorbic acid, 0.5 ml recombinant long R Insulin-Like growth factor-1 in aqueous solution (R^3 – IGF -1), recombinant human endothelial cell growth factor(rhEGF) in a buffered bovine serum albumin (BSA) saline solution, 0.5 ml recombinant human vascular endothelial growth factor (VEGF), 2.0 ml recombinant human fibroblast growth factor-B (rhFGF-B), 0.5 ml of heparin and 0.2 ml hydrocortisone (these supplements were Clonetics Clonetics® products). A 25 cm^2 flask with 5 ml of endothelial growth medium was placed in a humidified incubator at 37°C in 5% CO_2 in air for 30 minutes to achieve equilibrium between fluid and gas phase. A cryovial containing HUVEC (3.1.3.2) was thawed in warm water and emptied into the flask. The culture was incubated at 37°C with 5% CO_2 in air. The media in the flask was changed every two days until the cells had reached the required confluency.

3.1.3.2 Subculturing of HUVEC

All ingredients were brought to room temperature. Spent media was aspirated from the flask containing the HUVEC culture. The cells were then rinsed with 5 ml of HEPES- buffered saline (HEPES-BSS), to remove remaining media which contains complex proteins that neutralise the action of trypsin. Trypsinisation was performed as described in 3.1.1.2. The cells were then transferred to a 15 ml centrifuge tube. The flask was rinsed with the remaining 2 ml of HEPES-BSS to harvest any residual cells and the contents added to the centrifuge tube. To determine whether the cells have been successfully harvested the flask was observed under the inverted microscope and was expected to have less than 5% of cells left. The cell suspension was centrifuged at 220 x g for 5 minutes. The supernatant was aspirated leaving about 200 µl in the well, in which the cells were resuspended. The cells were then diluted with 2 ml of growth media.

The viable cell count was determined using trypan blue exclusion as described (3.1.1.3). The number of viable cells was then used to determine the total number of flasks to inoculate using the following equation:

$$\text{Total no. of flasks to inoculate} = \frac{\text{total no. of viable cells}}{\text{growth area x seeding density}}$$

The seeding density is recommended by the provider of the cell line (ATCC) and is defined as the number of cells allowed per surface area at the point of seeding. It differs per cell line. The growth area is the total surface area of the flask.

Five millilitres of growth media was transferred to each of the flasks and the required volume of cells suspension was added to each of them. The flasks were labelled with the passage number and cell type and incubated in a humidified chamber at 37°C with

5% CO_2 in air. Media was replenished every two days. When cells reached confluency they were trypsinised and then frozen in HUVEC freezing fluid (Appendix A) in 500 ml volumes.

3.2 CHEMOKINE AND ADHESION MOLECULE ASSAYS

3.2.1 Infection protocol

HUVEC were seeded in 24-well plates and allowed to become confluent prior to infection. The number and viability of cells was determined by trypsinising the cells in one well for each run and performing the trypan blue dye exclusion assay as described in 3.1.1.3. Cells were then infected with each of the *C. trachomatis* serovars at an MOI of 1. Three wells of HUVEC were also treated with 500 U of human recombinant tumor necrosis factor alpha (TNF-α) (formulation: 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, with BSA, 0.22 µM filtered, eBioscience) per ml which served as a positive control for the quantification of production of chemokines and the adhesion molecule (IL-8, MCP-1 and ICAM-1). The negative control comprised uninfected cells. Mock infected cells i.e. HUVEC treated with lysates of uninfected McCoy cells were also included as a control; this is because the chlamydia inoculum used for the experiments will have contained remnants of McCoy cells since it was propagated in McCoy cells.

The chlamydia strains were diluted with SPG to achieve the required MOI. Nine hundred microlitres of chlamydia growth medium (see Appendix B) was added to the wells containing HUVEC cells. HUVEC were inoculated separately with 100 µl of

each strain of *C. trachomatis*. Hundred microlitres of SPG was added to the negative control well. To facilitate infection chlamydia-cell contact was enforced by centrifugation at 1200 x g for 1 hour, followed by incubation for 1 hour at 37°C with 5% CO_2 in air. Following incubation, the medium was aspirated, cell monolayers were washed with Hanks' balanced salt solution (HBSS) (Clonetics®), and fresh chlamydia growth medium was added to the cultures. Uninfected, mock-infected, TNFα treated and infected cells were incubated at 37°C with 5% CO_2 in air before chemokine assays were performed.

After 24 hours, supernatants were collected and stored at -80°C until used to measure levels IL-8 and MCP-1 by commercially available enzyme-linked immunosorbent assay (ELISA) kits.

3.2.2 Monocyte Chemotactic Protein – 1 ELISA

Measurement of MCP-1 production by chlamydia infected HUVEC was carried out using the MCP-1 ELISA kit (DIACLONE) according to the manufacturer's instructions. All reagents were made according to the manufacturer's instructions (see Appendix C) Two-fold dilutions of the standard were prepared in duplicate. All experimental wells were in triplicates. Eighty microlitre of assay buffer (provided with the kit) was added to the experimental wells followed by 20 µl supernatant from the chlamydia infected cells or controls. Fifty microlitre of HRP conjugate was then added to all the wells. The plate was then covered and incubated at room temperature (18°C-25°C) for 1 hour. The wells were emptied and washed thrice with wash buffer. After the last wash the plate was blotted with paper towel. One hundred microlitre of

tetramethylbenzidine (TMB) Substrate Solution was added to all wells and the plate was covered with aluminium foil and incubated for 8 minutes at room temperature. The reaction was stopped by adding 100µl of stop solution to each well. Absorbance readings were measured at 450 nm as the primary wavelength and 620 nm as the reference wave length, using an Anthos 2010 ELISA reader.

3.2.3 Interleukin -8 ELISA

The IL-8 ELISA was also carried out according to the manufacturer's instructions using the IL-8 ELISA kit. The procedure was the same as the MCP-1 one except that a 100 µl of supernatant from each serovar was added to sample wells and 100 µl of reconstituted control vial was added in duplicate to control wells H1 and H2. One hundred microlitres of diluent was also added to the blank wells. Biotinylated anti IL-8 was prepared and 50 µl of this solution was added to all wells. The cells were then covered with a plate cover and incubated for 1 hour at room temperature. Streptavidin-HRP solution was prepared and 100 µl of this was added to all wells, the plate was covered and incubated for 30 minutes at room temperature. Unlike the MCP-1 ELISA, after addition of TMB solution, the plate was incubated for 12 minutes instead of 8 minutes.

3.2.4 Cell surface Intercellular Cell Adhesion Molecule-1 ELISA

The same infection protocol as for the chemokine assays was carried out (see 3.2.1). The difference was that 96-well plates were used, so 10 µl of each *C.trachomatis* serovars prepared in 3.1.2 was used to infect HUVEC at an MOI of 1. The adhesion

molecule ELISA was carried out as indicated by Al-Numani *et al*. (2003) and Krüll *et al.* (1997) as follows. All incubation steps were carried out at room temperature.

After incubation the serovars and controls were aspirated and the monolayer was fixed with 1% paraformaldehyde for 20 minutes. The paraformaldehyde was aspirated and cells were washed with PBS. The cells were then blocked with 1% FCS in PBS. An antibody against the adhesion molecule ICAM-1 (anti-human1CAM-1) purchased from R&D Systems, Minneapolis, USA, was the added at a concentration of 50 µg/ml and the plate was incubated for 1 hour at room temperature. Plates were washed three times with PBS and then exposed to horseradish peroxidase-conjugated goat antimouse IgG antibody(R& D Systems, Minneapolis, USA) for 30 minutes. Detection of the bound enzyme was carried by incubating with a 1:1 solution of TMB and hydrogen peroxide for 10-20 minutes. Absorbance was read at 450 nm.

3.2.5 Calculation of Results

The average absorbance of the standards and samples was calculated for the IL-8 and MCP-1 ELISAs. The standard curve was then created by plotting average absorbance of standards against the respective cytokine concentration. The best fit line was then drawn through the points. This curve was used to determine the concentration of the mean absorbance values of the samples. Since experiments had varying inoculation concentration, all results were expressed per 100 cells. For the ICAM-1 ELISA, the average absorbance of each serovar was calculated. All the results were then analysed using one way analysis of variance with Turkey's post test (Graphpad Instat) as described in 3.5.

3.3 TRANSENDOTHELIAL MIGRATION ASSAY

Transendothelial migration of leukocytes is a complex multistep process initiated by inflammatory mediators. It occurs when leukocytes migrate through the endothelium into the infected tissue. The assay was carried out as described by Molestina *et al.* (1999). It quantifies the number of neutrophils and monocytes that migrate during a fixed time period through a confluent monolayer of endothelial cells. The transendothelial migration induced by *C. trachomatis* serovars was investigated through a series of steps as described below.

HUVEC were seeded on 6.5-mm Transwell-Clear inserts at a density of 4×10^5 cells/insert. These inserts were placed in the tissue culture wells containing growth medium and these were incubated to allowed the cells to reach confluency before infection. The formation of confluent monolayers was verified by silver nitrate stain and microscopic examination under a 40× objective.

3.3.1 Silver Nitrate staining

This was carried out on one transwell. Growth media was discarded from the well and the monolayer was rinsed with RPMI containing 10% FBS. The monolayer was treated with 0.25% silver nitrate ($AgNO_3$) (kindly donated by the Department of Mycotoxins) in distilled water for 40 seconds, rinsed with 5% glucose, then fixed with 4% formaldehyde. The stained monolayer was exposed to UV light by putting the transwell in the safety cabinet with the UV light on for 5 minutes to intensify the stain. The cells were then observed under an inverted microscope with a 40x objective

lens. The confluent monolayer was photographed using the Olympus CMOS color camera with Analyse® getIT software.

3.3.2 Infection protocol

HUVEC monolayers in 6.5-mm transwells were infected separately with each isolate of *C. trachomatis* suspended in inoculation medium. The cells were inoculated with 4 × 10^5 inclusion-forming units (IFU) per well, resulting in a MOI of 1. The inoculation media was the same as the one used to inoculate McCoy cells except cycloheximide was not added. Media was removed from transwells and they were washed with 100 µl of PBS. The PBS was discarded and replaced with 90 µl of chlamydia growth media. Ten microlitres of SPG (pH 7.5) containing the appropriate amount of each serovar of *C. trachomatis* to achieve an MOI of 1 was added the appropriate wells. Also included were the negative controls consisting of sterile SPG and SPG containing lysed McCoy cells as well as a positive control consisting of TNFα. Infection was performed by incubation for 2 h at 37°C with 5% CO_2 in 95% air. Following incubation, the medium was aspirated, the cell monolayers were washed with Hanks' balanced salt solution (HBSS) (Clonetics®) and fresh inoculation medium was added to the cultures. The plates were then incubated for 24 hours before the transendothelial migration assay.

3.3.3 Isolation of human neutrophils and monocytes

For isolation of peripheral blood mononuclear cells (PBMC) and neutrophils, 50 ml of blood was collected from healthy volunteers using a sterile syringe. Blood was collected in 9 ml EDTA tubes.

PBMC and neutrophils were isolated from whole blood using Histopaque gradient density centrifugation under sterile conditions. Histopaque-1077 and 1199 were warmed to room temperature before use. Twelve millilitres of Histopaque 1119 was added to a 50 ml centrifuge tube. An equal volume of Histopaque-1077 was carefully layered onto the Histopaque-1119. Twenty-four millilitres of whole blood was then carefully layered onto the upper Histopaque layer. The tube and its contents were centrifuged at 700 x g for 30 minutes at room temperature. Six distinct layers were observed after centrifugation. Layer A contained the PBMC and layer B contained the neutrophils (figure7).

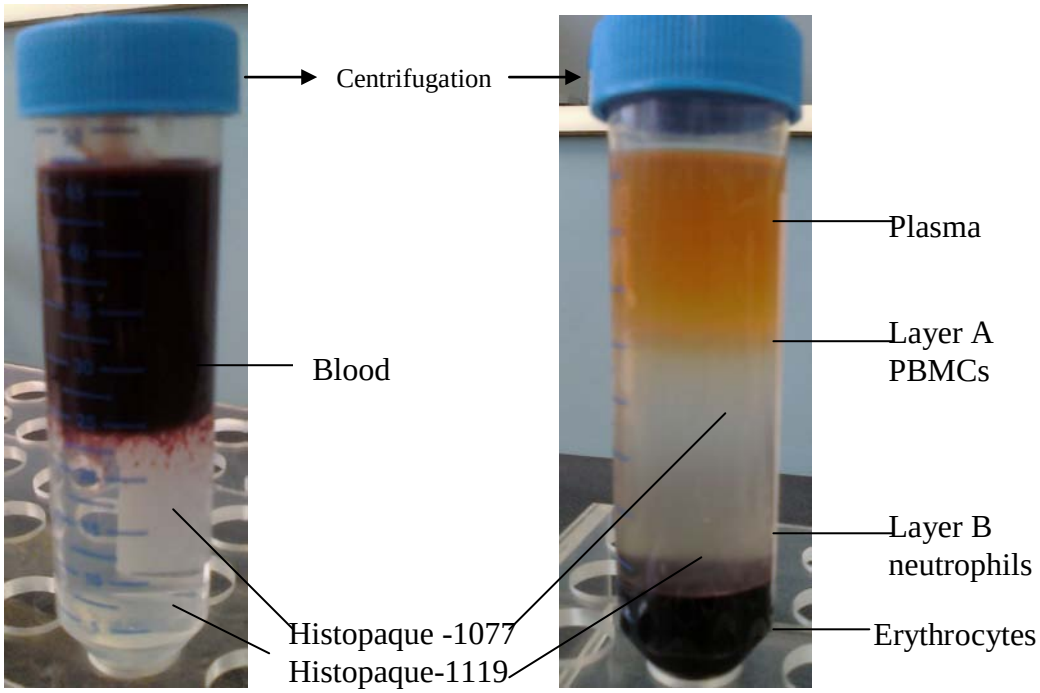


Figure 7: Demonstration of the layers obtained before and after centrifugation during monocytes and neutrophils isolation.

The top layer was aspirated to within 0.5 cm of layer A using a Pasteur pipette and discarded. The layer A cells were transferred into a 15 ml centrifuge tube labelled

PBMC. The Histopaque-1077 layer was aspirated and discarded to within 0.5 cm of layer B. The layer B cells were transferred to a 15 ml centrifuge tube labelled neutrophils. The cells in both tubes were washed with isotonic PBS. Ten millilitres PBS was added and the tubes were centrifuged for 10 minutes at 200 x g. After centrifugation the supernatant was discarded and the cells were resuspended by gentle aspiration with a Pasteur pipette. Cells were washed 3 time altogether. The cells were then resuspended in chlamydia growth medium (CGM). To determine the concentration of cells a cell count was carried out using the trypan blue dye exclusion assay and a haemocytometer.

3.3.4 Transendothelial migration assay

After 24 hours of infection, the media from the upper and lower chambers of the transwells was aspirated. The endothelial layer was then washed three times with HBSS. To one 24 well plate set up for the neutrophil migration assay, neutrophils were added to the upper chamber at a density of 4×10^6. Fresh medium was also added to the lower chambers. The plate was then incubated for 30 minutes at 37 ^oC in 5% CO_2 in air. To the plate labelled monocytes, monocytes were added to the upper chamber at the same density as neutrophils. The lower chambers were also filled with 600 µl of fresh medium. This plate was incubated for one hour. Cells were added to all transwells including the control transwells.

Following incubation, the upper chambers from both plates were removed. Cells counts were done on each well (lower chamber media) immediately using the haemocytometer and the trpyan blue dye exclusion assay. Each well was swirled

before taking out the required amount of cells to allow equal distribution of cells throughout the well. After the counts the number of cells which migrated was determined using the formula in 3.1.1.3 which gave the concentration of cells in 1 ml of media. To find the number of cells in 600 µl which is the amount of media in the lower chamber the concentration was multiplied by 0.6 ml.

3.4 LACTATE DEHYDROGENASE RELEASE CYTOTOXICITY ASSAY

3.4.1 Infection protocol

HUVEC were seeded on 24-well plates at a density of 4.0 x 10^5 and allowed to adhere until confluent. Cells were monitored by doing cell counts until they reached a density of 4.0 - 4.5 x 10^5 . Inoculation of cells in 24 well plates for the cytotoxicity assay was carried out as for the chemokine assays with MOI of 1. In addition to the experimental wells, 500 U of TNFα was used as a positive control, mock infected and uninfected cells were also included as controls. After infection the plate was incubated for 24 hours at 37°C with 5% CO_2 in air.

3.4.2 Lactate Dehydrogenase Release Assay

When the cell membrane looses its integrety, the cell releases the cytosolic enzyme lactate dehydrogenase (LDH). The CytoTox 96 ® Non-Radioactive Cytotoxicity Assay kit (Promega) was used to quantify the level of LDH released into the supernatant by a coupled enzymatic assay. This assay converts tetrazolium salt into a red formazan product. The intensity of the colour change is directly proportional to

the number of lysed cells (Moravec, 1994). The assay was carried out according to the manufacturer's instructions, except that the lysis solution was prepared in-house. The lysis solution supplied with the kit (9% Triton X-100) was insufficient to produce 100% LDH release in this experiment, but 15% Triton X-100 in triple distilled water did. The Substrate mix was reconstituted using the assay buffer. The assay buffer was allowed to come to room temperature in the dark. Twelve millilitres of assay buffer was then added to a bottle of Substrate mix. The solution was mixed gently by inverting to dissolve the substrate. Unused reconstituted substrate mix was tightly capped and stored at -20 °C for ≤ 8 weeks.

Forty-five minutes before collection of supernatants, 100 µl of lysis solution was added to a set of triplicate wells with HUVEC monolayers and designated Target Cell Maximum LDH Release. Another 100 µl was added to triplicate of wells with media only i.e. without cells. This was to correct for the volume increase caused by the addition of lysis solution and was designated Volume Correction Control. Also included was a triplicate of wells containing culture medium to correct for contributions caused by phenol red and LDH activity that may be present in serum-containing culture medium and this was designated Culture medium Background. After 24 hours of incubation the plate was centrifuged at 250 x g for 4 minutes. Supernatants were collected and used to determine the cytotoxicity levels.

Fifty microlitres of supernatants of each sample was transferred to the corresponding well on a 96 well plate. Fifty microlitres of the reconstituted substrate mix was added to each well. The plate was covered with aluminium foil and incubated at room

temperature for 30 minutes. The reaction was stopped by adding 50 µl of stop solution to each well. Absorbance was read immediately at 490 nm using a spectrophotometer.

3.4.3 Calculation of Results

The culture medium background average absorbance was subtracted from all values of experimental and the target cell spontaneous LDH release, i.e. the negative control with uninfected cells. The Volume Correction Control average absorbance was subtracted from the average absorbance values of the Target Cell Maximum LDH Release Control. The above corrected values were then used to calculate the % cytotoxicity for each experimental well using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental-Target Spontaneous}}{\text{Target Maximum-Target Spontaneous}} \times 100 \quad \text{(Appendix D)}$$

Results were then analysed using one way analysis of variance with Turkey's post test (Graphpad Instat; see 3.5).

3.5 STATISTICAL ANALYSIS

The ELISA, transendothelial migration and cytotoxicity assays results were statistically analysed using Graphpad Instat version 3.00(Appendix E). Significance between standard deviation of groups was determined using Bartlett's test. Gaussion distribution was tested using the Kolmogorov and Smirnov to confirm whether data was parametric or not. When data was confirmed to be parametric then Oneway Analysis of Variance (ANOVA) was used. The transendothelial migration assay

results did not pass the normality test because there were few values (3 values per serovar).

3.6 APOPTOSIS ASSAYS

Analysis of cell death was carried out using two methods, i.e the modified CaspGLOW™ Flourescein Caspase Staining kit (BioVision - BiocomBiotech) and the modified DeadEnd™ Colorimetric TUNEL System from Promega.

3.6.1 CaspGLOW™ Flourescein Caspase Staining Assay

Caspases (2, 3, 6, 7, 8, 9 & 10) are involved in apoptosis (Creagh *et al*., 2003). The CaspGLOW™ Fluorescein Caspase Staining kit (BioVision - BiocomBiotech) is based on conjugation and proteolysis of tetrapeptide substrates with caspase cleavage sites. This assay uses the caspase family inhibitor Val-Ala-Asp-α-fluoromethylketone (VAD-FMK) conjugated to fluorescein isothiocynate (FITC) forming a product known as FITC-VAD-FMK as a marker.

3.6.1.1 Infection protocol

HUVEC were grown on glass coverslips in 24-well tissue culture plates and allowed to reach confluency. After a cell count with trypan blue dye, cells were then infected with *C. trachomatis* serovars L1, L2, L3 and E at a MOI of 1. Infection was carried out as for the chemokine assays. Also included were the positive control TNFα, the negative control where only SPG (pH 7.5) was added and mock infected wells. For

the assay kit, etoposide at a concentration of 20 µM was used as a positive control. The etoposide was added to wells containing HUVEC 4 hours before the assay. Infected cells were incubated for 24 hours after which the apoptosis assay was carried as described below:

3.6.1.2 Analysis of Cell death

Analysis of cell death was carried out using the CaspGLOW^{TM} Flourescein Caspase Staining kit (Biovision-BiocomBiotech) with modification because the kit was designed for suspension cells whereas HUVEC are not. The kit was modified as follows: media was aspirated from the wells and 300µl of CGM + 1 µl FITC-VAD-FMK was added to the wells. The cells were incubated for 0.5-1 hour. Media was aspirated and cells were washed with wash buffer for 5 minutes. The cells were then fixed with10% formaldehyde in wash buffer for 5 minutes. A final wash with wash buffer for 5 minutes was done. Slides were then prepared by placing coverslips cell side down on glass slides with a drop of buffer. Cells were observed under a Nikon eclipse E600 fluorescent microscope and photographed using a Nikon ColorView Soft Imaging System digital camera. Comparisons were made of the different serovars and the controls.

3.6.2 DeadEnd^{TM} Colorimetric TUNEL System Assay

The DeadEnd^{TM} Colorimetric TUNEL System (Promega) was used to analyse cell death by measuring one of the characteristics of apoptotic cells which is nuclear DNA fragmentation. The terminal deoxynucleotidyl transferase recombinant (rTDT)

enzyme is used to incorporate the 3`-OH DNA ends with a biotinylated nucleotide. Horse radish peroxidase (HRP) labelled streptavidin binds to the biotin on the nucleotide and the HRP label is detected using diaminobenzidine (DAB) stain solution. This results in dark brown apoptotic nuclei.

3.6.2.1 Infection protocol

Seeding of HUVEC and infection with chlamydia was carried out as for the TUNEL assay. The TUNEL positive and negative controls were provided with the kit. The procedure was carried out according the manufacturers instructions and modifications by Joubert, (2009) as follows:

3.6.2.2 Analysis of Cell death

The second analysis of cell death was carried out using the DeadEnd™ Colorimetric TUNEL System (Promega). The assay was carried out according to the manufacture's instructions wit some modifications to fit our setting.

Instead of processing cells on slides as stated by the manufacturer, cells were processed on coverslips which were within the wells of a 24 well plate. The coverslips were only mounted to glass slides after the staining procedure. Processing of the cells was done at room temperature unless otherwise stated. The procedure was as follows: Cells were washed with PBS and fixed with 4% formalin in PBS for 25 min instead of the recommended 10% formalin in PBS by the manufacturer. The cells were then washed twice with PBS for 5 min. Permeabilization was done by incubation with 0.4% Triton® X-100 in PBS for 10 min 37°C instead of the 0.2% recommended by the

manufacturer. Prior work done in the department of Medical Microbiology, University of Kwa-Zulu Natal by Joubert in 2009 had shown that the recommended concentration of Triton® X-100 did not sufficiently permeabilize the HaCaT keratinocytes. Cells were washed twice with PBS for 5 min. The DNase TUNEL positive control (10units/ml RQ1 DNase) was then treated by the following steps: washed with 200 µl DNase I buffer for 5 min, treated with 200 µl RQ1 RNase free DNase (10 units / ml) (Promega) in DNase I buffer for 10 min at 37°C, rinsed 4 times with tdH_2O and then washed with PBS for 5 min. Equilibration was done with 100 µl equilibration buffer for 5-10 minutes. Recombinant Terminal Deoxynuclotidyl Transferase (rTdT) reaction mix was prepared by mixing the following components per well: 98 µl equilibration buffer, 1 µl biotinylated nucleotide mix and 1 µl rTdT enzyme or 1 µl autoclaved tdH_2O for the TUNEL negative control. Cells were labelled with 100 µl rTdT reaction mix for 60 min at 37°C. To prevent evaporation and ascertain that the reagent is evenly distributed during the labelling procedure each glass coverslip was covered with a plastic coverslip. The plastic coverslips were removed and the reaction stopped with 200 µl 2X Sodium-Saline Citrate (SSC) for 15 min. washing was done thrice with PBS for 5 min. Endogenous peroxidases was blocked with 0.3% hydrogen peroxide for 4 min. Then another wash three times with PBS for 5 min was done. Binding with 200 µl streptavidin horse radish peroxidase (HRP) (1:500 in PBS) for 30 min and washing thrice with PBS (pH 7.4, Oxoid) for 5 min then followed. Diaminobenzidine (DAB) stain solution was then prepared by adding 950 µl tdH_2O, 50 µl 20X DAB substrate buffer, 50 µl DAB 20X chromagen and 50 µl 20X hydrogen peroxide. The 100 µl DAB solution was used to stain each glass coverslip for 15 minutes covered with a plastic coverslip for reasons already

mentioned above. Plastic coverslips were then removed and the monolayer was rinsed 5 times with tdH_2O.

Counterstaining of the slides with the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit was done at the end of the TUNEL assay staining procedure. Briefly, the slides were moistened with PBS (pH 7.4, Oxoid) for 5 min and stained with 30 µl stain reagent for 30 min at 37°C in a humidified chamber with shaking every 10 minutes. The monolayer was then washed with tdH_2O for 10 sec. Glass coverslips were removed from the wells and blotted on a paper towel. Coverslips were finally mounted to glass slide with DPX mounting fluid and left to dry.

The slides were viewed with a Nikon eclipse E600 fluorescent microscope. Bright field was used to examine identify cells with fragmented DNA and DAB positive nuclei; The mode was then switched to fluorescence (excitation wavelength 450-490 nm; emission wavelength 520 nm) to identify the apple green *C. trachomatis* as confirmation that the cell were indeed infected with *C. trachomatis*. The slides were photographed using the Nikon ColorView Soft Imaging System digital camera. Comparison of apoptotic cells was then done amongst serovars

CHAPTER 4: RESULTS

4.1 Infection of HUVEC with chlamydial serovars

All *Chlamydia trachomatis* serovars (L1, L2, L3 and E) successfully infected and replicated in HUVEC (Figure 8).

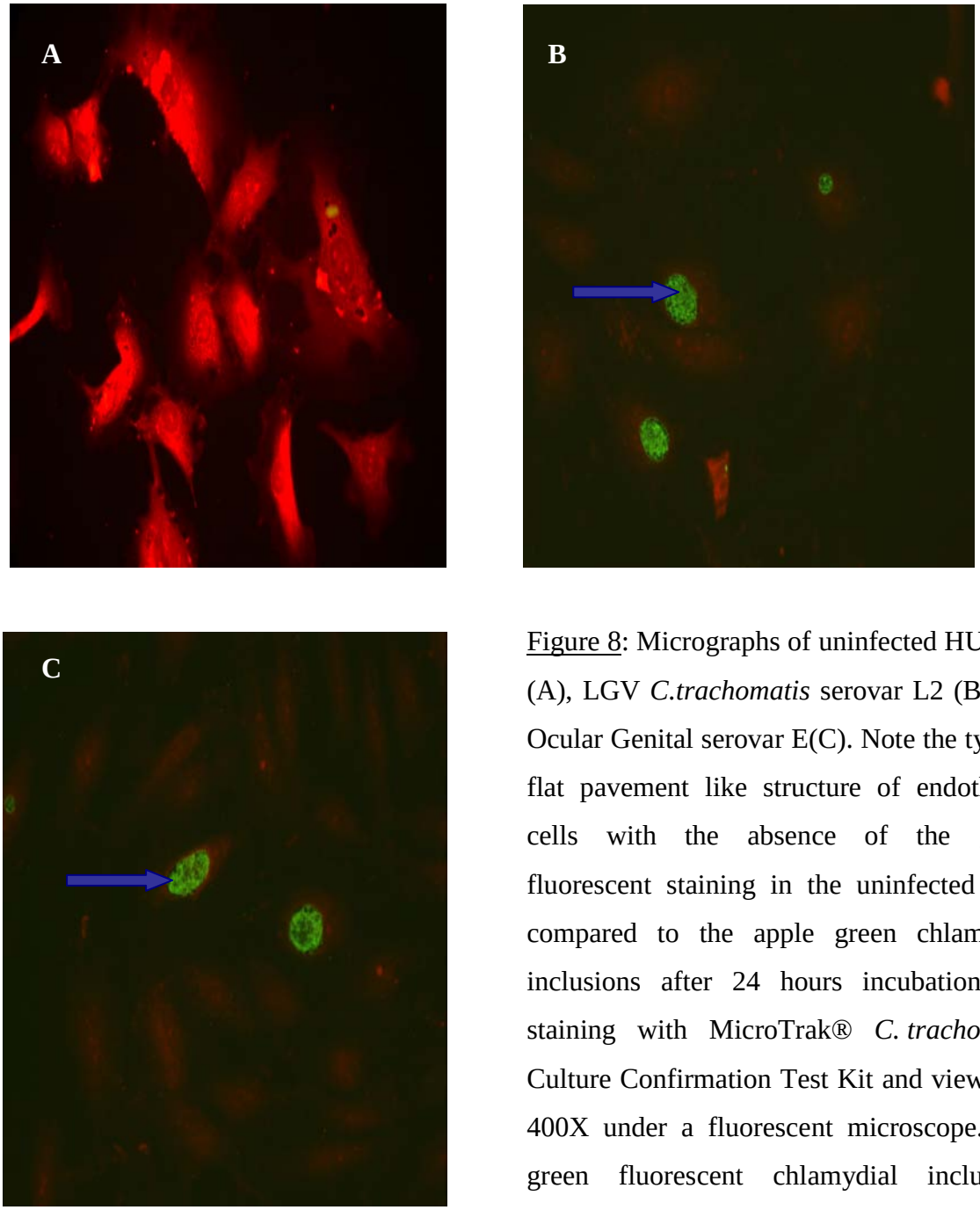


Figure 8: Micrographs of uninfected HUVEC (A), LGV *C.trachomatis* serovar L2 (B) and Ocular Genital serovar E(C). Note the typical flat pavement like structure of endothelial cells with the absence of the green fluorescent staining in the uninfected cells compared to the apple green chlamydial inclusions after 24 hours incubation and staining with MicroTrak® *C. trachomatis* Culture Confirmation Test Kit and viewed at 400X under a fluorescent microscope. The green fluorescent chlamydial inclusions confirmed that *C. trachomatis* did infect HUVEC(arrows).

4.2 Chemokines and Adhesion Molecules Assays

4.2.1 Chemokines (IL-8 and MCP-1) stimulation by *C. trachomatis* infected HUVEC.

IL8 production by the different serovars is shown in figure 9. Only L3 stimulated significantly higher production of IL-8 ($p<0.001$) as compared to the negative controls (cells mock-infected with lysed uninfected McCoy cells or unexposed HUVEC cells) (Appendix E). There was no significant difference in the production of IL-8 between the other LGV biovars (L1 and L2) and ocular genital serovar E and the negative controls ($p>0.05$). L3 also stimulated a significantly higher production of IL8 as compared to the other serovars (L1, L2 and E, with $p<0.001$, $p<0.001$ and $p< 0.01$ respectively (Appendix E).

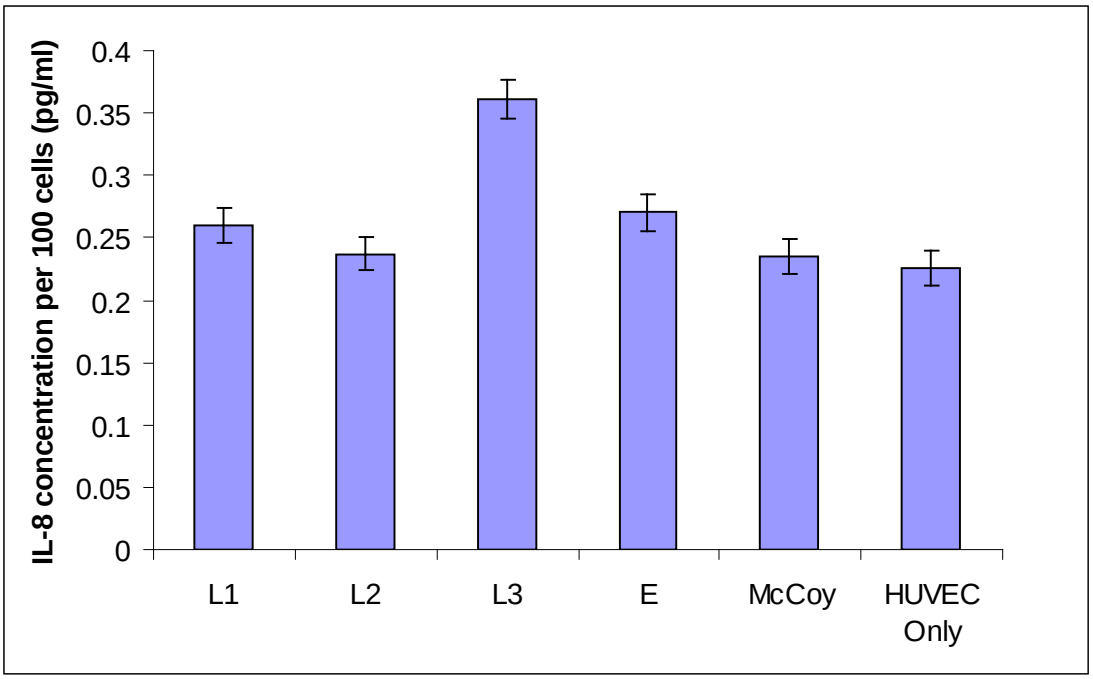


Figure 9: Mean levels of IL-8 induced in HUVEC infected with different *C. trachomatis* serovars and controls after 24 hr of incubation, expressed per 100 cells. Bars indicate the means ± standard errors of the means of three separate experiments performed in triplicates.

MCP-1 production by chlamydia exposed HUVEC is shown in figure 10. L3 stimulated significantly higher amount of MCP-1 compared to the negative and the mock infected cells (at $p<0.001$ and $p<0.05$ respectively). There was also no significant difference in the levels of MCP-1 produced between the remaining LGV (L1 and L2) and the OG strain. The LGV strains also did not differ significantly amongst each other in the production of MCP-1 at a p value of > 0.05(Appendix E).

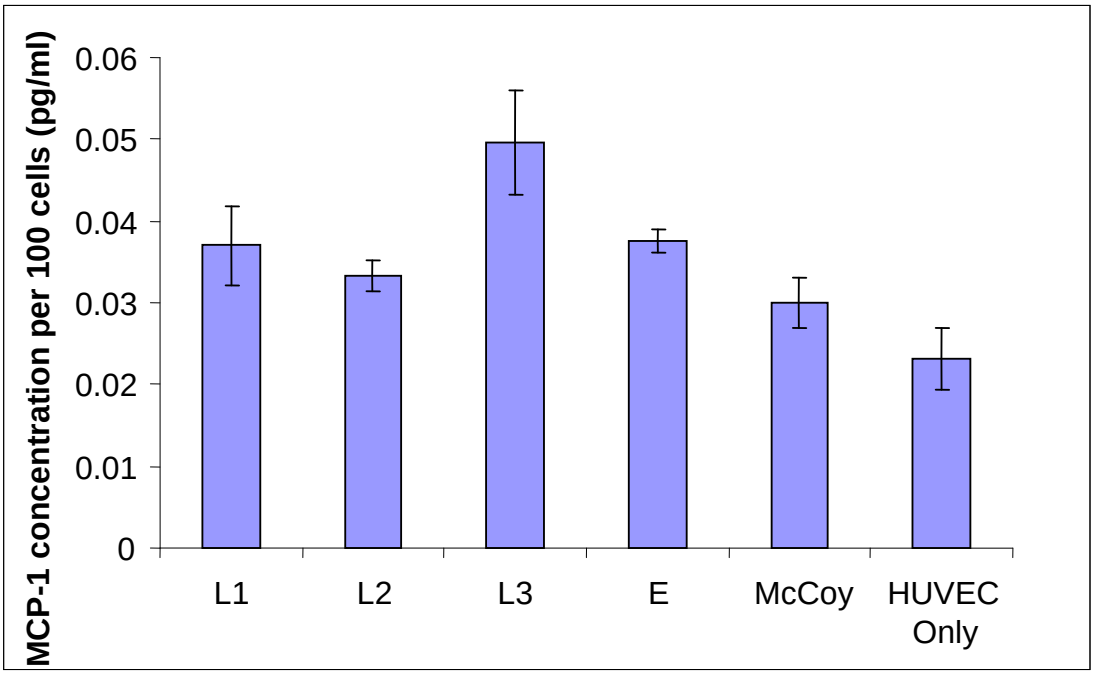


Figure 10: Mean levels of MCP-1 induced in HUVEC infected with different *C. trachomatis* serovars and controls after 24 hr of incubation, expressed per 100 cells. Bars indicate the means ± standard errors of the means of three separate experiments performed in triplicates.

4.2.2 Adhesion molecule (ICAM-1) stimulation on *C. trachomatis* infected HUVEC.

After 24 hour incubation of *C. trachomatis* infected HUVEC, the level of expression of ICAM-1 was measured on the 96 well plates. Only L3 showed a significant expression of ICAM-1 compared to the negative control and cells exposed to

remnants of McCoy ($p<0.05$ and $p<0.01$ respectively). However, the remaining LGV serovars and the E serovar showed no significant difference in the expression of ICAM-1 when compared to the controls (all at $p>0.05$). L3 also significantly expressed more ICAM-1 adhesion receptors on HUVEC as compared to the L2 ($p<0.05$). There was no significant difference between L1, L2 and E at p value of $p>0.05$(Appendix E).

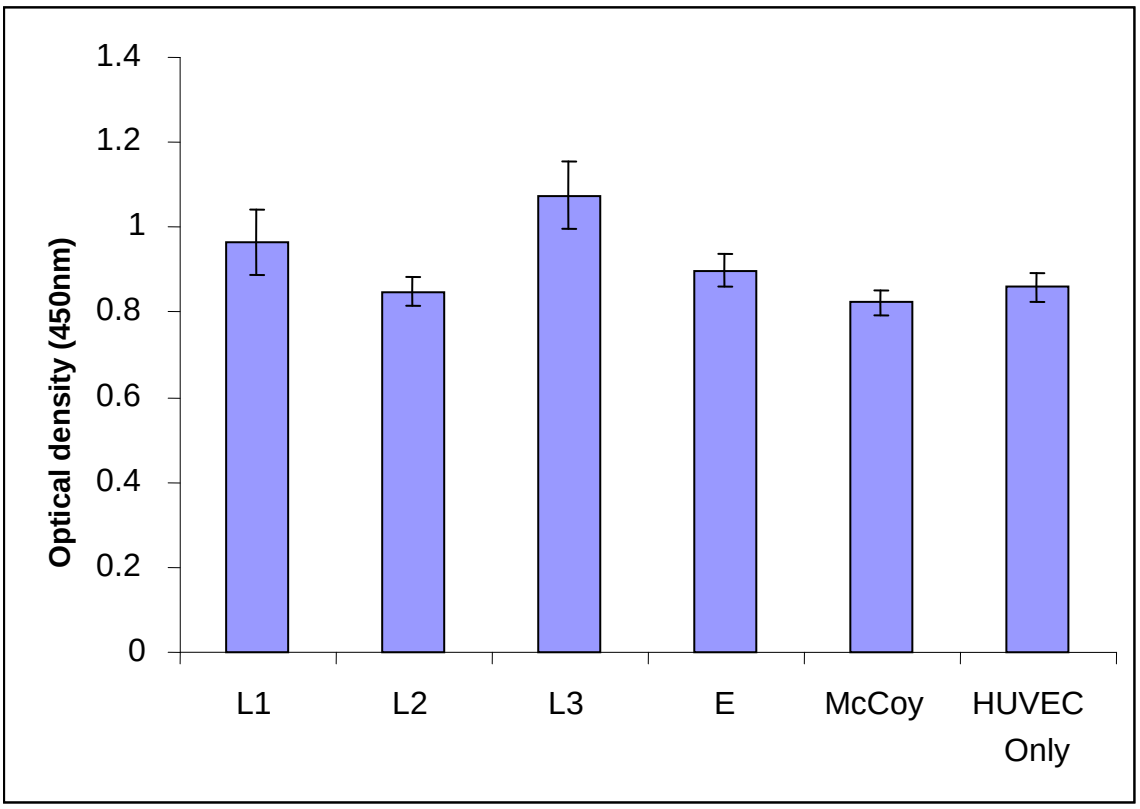


Figure 11: Mean levels of ICAM-1 expression by HUVEC infected with *C. trachomatis* serovars infected HUVEC and controls. Quantification was done after 24 hr of incubation. Bars indicate the means ± standard errors of the means of three separate experiments performed in triplicates.

4.3. Transendothelial migration assay

Three separate experiments were done for the transendothelial migration of neutrophils and monocytes and the results are shown in figure 13. Although the differences in the number of migrated neutrophils between untreated HUVEC, mock infected HUVEC and HUVEC infected with chlamydia serovars (Appendix E) did not reach statistical significance (> 0.05), there appeared to be a trend towards a stronger neutrophil migration with the LGV serovars. L3 induced the highest level of neutrophil migration compared to the other serovars. The OG serovar E showed the same migration level as the mock infected cells.

Statistical analysis of monocytes transendothelial migration showed that HUVEC infected with L3 had significally higher amount of migrated monocytes than the controls. L2 infected HUVEC also had a significally higher number of migrated monocytes compared to the negative control. All the LGV serovars had a higher number of migrated monocytes than the OG serovar. The OG serovar E did not show any significant stimulation of migration of monocytes as compared to the negative and mock-infected controls. All comparison was at a p value of >0.05 (Appendix E).

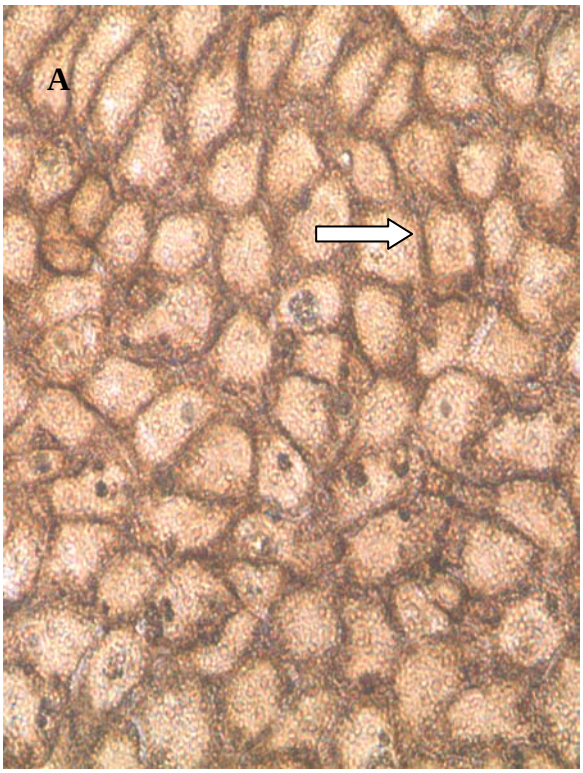


Figure 12: A confluent monolayer of HUVEC grown in transwells for transedothelial migration assays.(A) Cells were stained using the silver nitrate stain ,which form silver grains at the intercellular junctions (arrow) and observation was done under an inverted light microscope to confirm that the cells are confluent. Magnification X200.

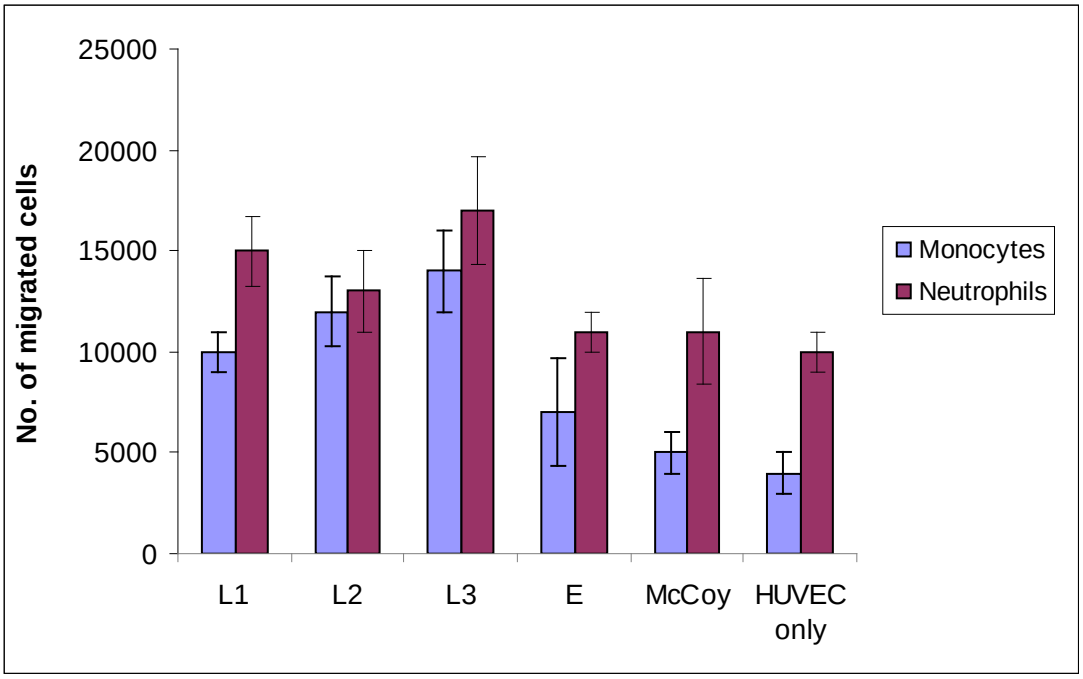


Figure 13: Mean number of migrated monocytes and neutrophils through HUVEC infected with different *C. trachomatis* serovars and controls after 24 hr of incubation. Bars indicate the means ± standard errors of three separate experiments.

4.4. Cytotoxicity assay

Cell death due to necrosis of HUVEC infected with *C. trachomatis* serovars was analysed using the LDH assay kit. The average percentage cytotoxicity of the serovars and controls is shown in figure 14. Only L3 had significantly higher percentage cytotoxity than the negative control ($p<0.05$) but not with the mock infected cells ($p>0.05$). There was no significant difference between the controls and the rest of the LGV serovars and the OG serovar E, all at p value of >0.05. The cytotoxity effect of OG serovar E on HUVEC was not significantly different to the LGV serovars (all at a p value of >0.05) (Appendix E).

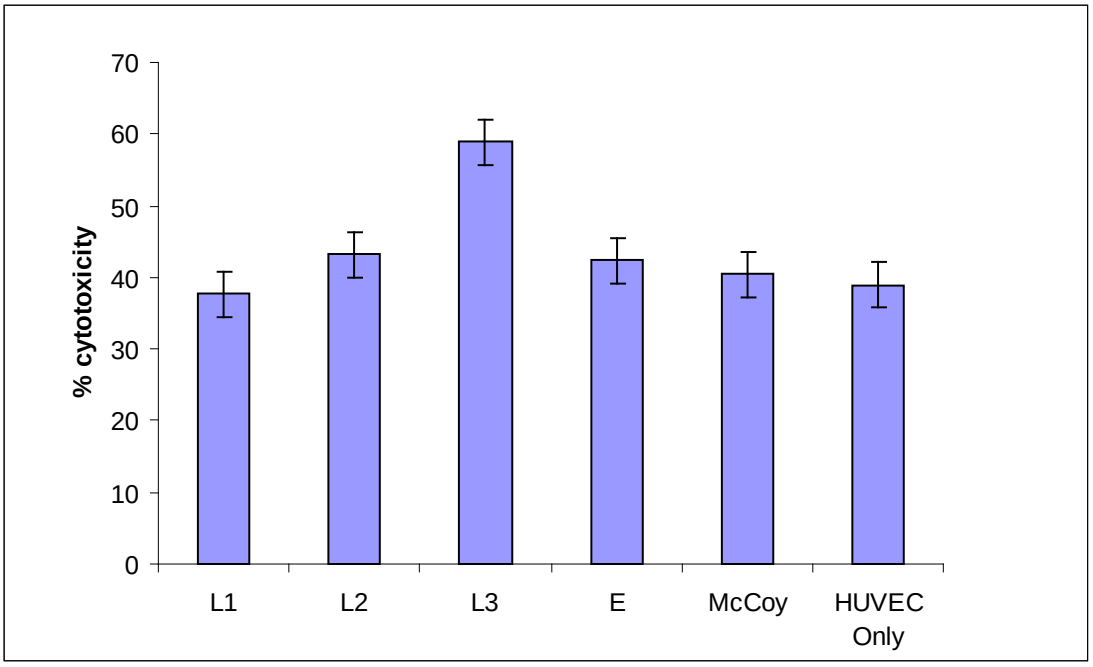


Figure 14: Mean levels of percentage cytotoxicty of HUVEC infected with different *C. trachomatis* serovars and controls after 24 hr of incubation. Cytotoxicty was measured by quantifying the level of LDH released from cells. Bars indicate the means ± standard errors of the means of three separate experiments performed in triplicates.

4.5 Apoptosis assay

4.5.1 DeadEnd™ Colorimetric TUNEL System Assay

TUNEL assay was used to assess cell death on endothelial cells infected with HUVEC serovars.The DNase treated positive control exhibited some brown stained nuclei(figure 15A), whereas the TUNEL negative (TdT free) control showed no dead cells(figure 15B). The uninfected negative control (figure 15C) showed some DAB positive nuclei, however these were darker and smaller than those observed in the DNase positive treated control.

Micrographs of HUVEC infected with different serovars were taken, at bright field to show the TUNEL stained nucleus and the same field of view was used to take micrographs of chlamydial infected cells with fluorescence view (figure 16A, B, C, D and figure 17A, B, C and D). The small brown stained DAB positive nuclei similar to the ones found on the uninfected negative controls were observed in all the serovars which might be attributed to normal cell turnover hence apoptosis (indicated by white arrows).

Comparison of the bright field micrographs with fluorescent micrographs showed a light brown staining where the infected cells are positioned(blue circles) which might mean that the DNA was starting to fragment, hence if given enough time there might have been apoptosis since this assay was done after only 24 hours of incubation. There appeared to be no difference between numbers of dark brown stained DAB positive nuclei on uninfected cells compared to the chlamydia infected cells. The only difference was the presence of the faint light brown larger stains on infected cells which were not present on uninfected cells. There appeared to be no difference in the

number and appearance of DAB stained nuclei amongst the LGV (L1-L3) and Ocular genital serovars (E)

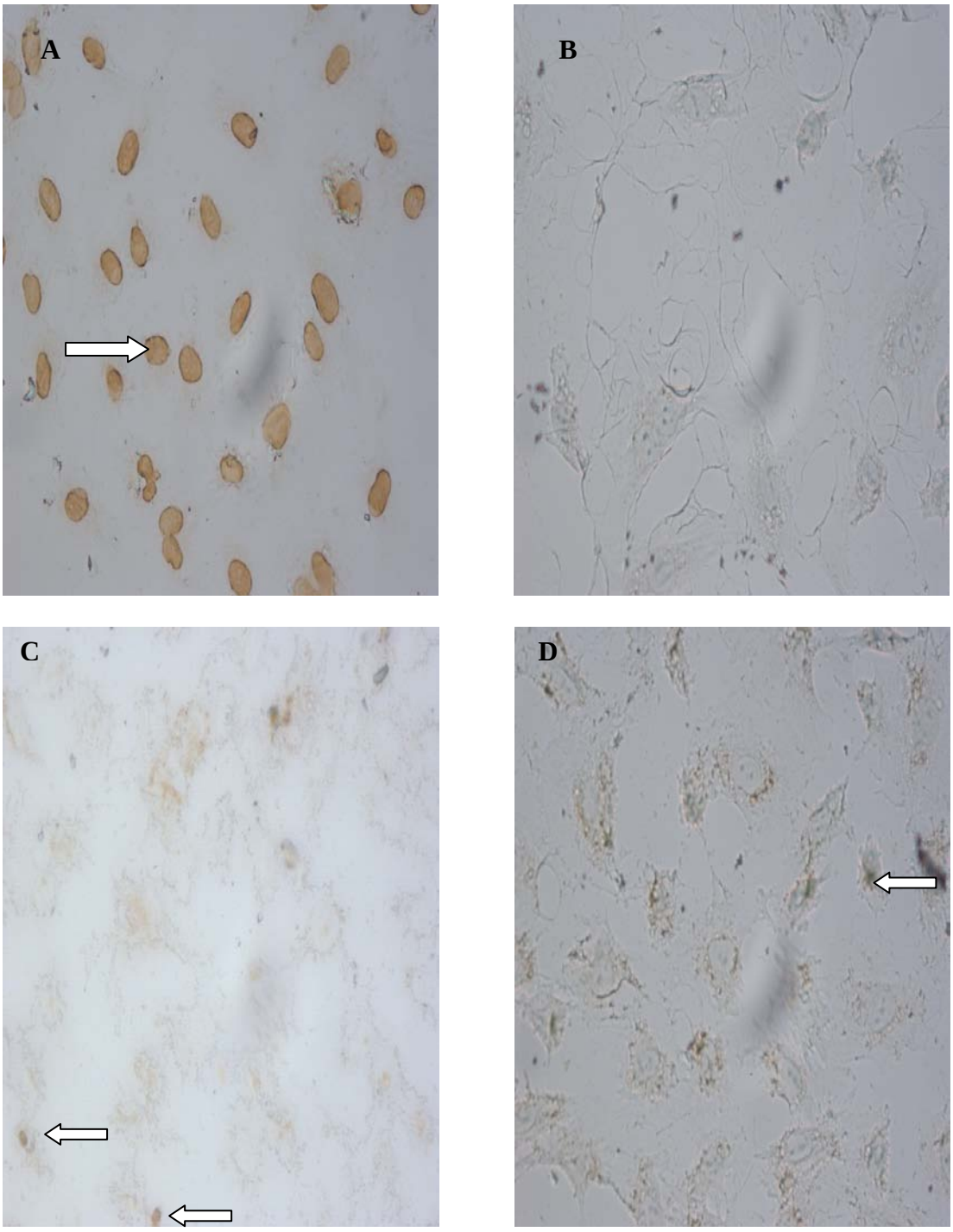


Figure 15: HUVEC monolayers TUNEL assay controls. (A) Positive control treated with DNAse. Note the dark brown stained nuclei indicating of apoptosis. (B)-TUNEL negative control (TdT free) with no apoptotic cells (C) uninfected control and (D) HUVEC exposed to McCoy remnants, the latter two had a few DAB positive nuclei. Maginification x400

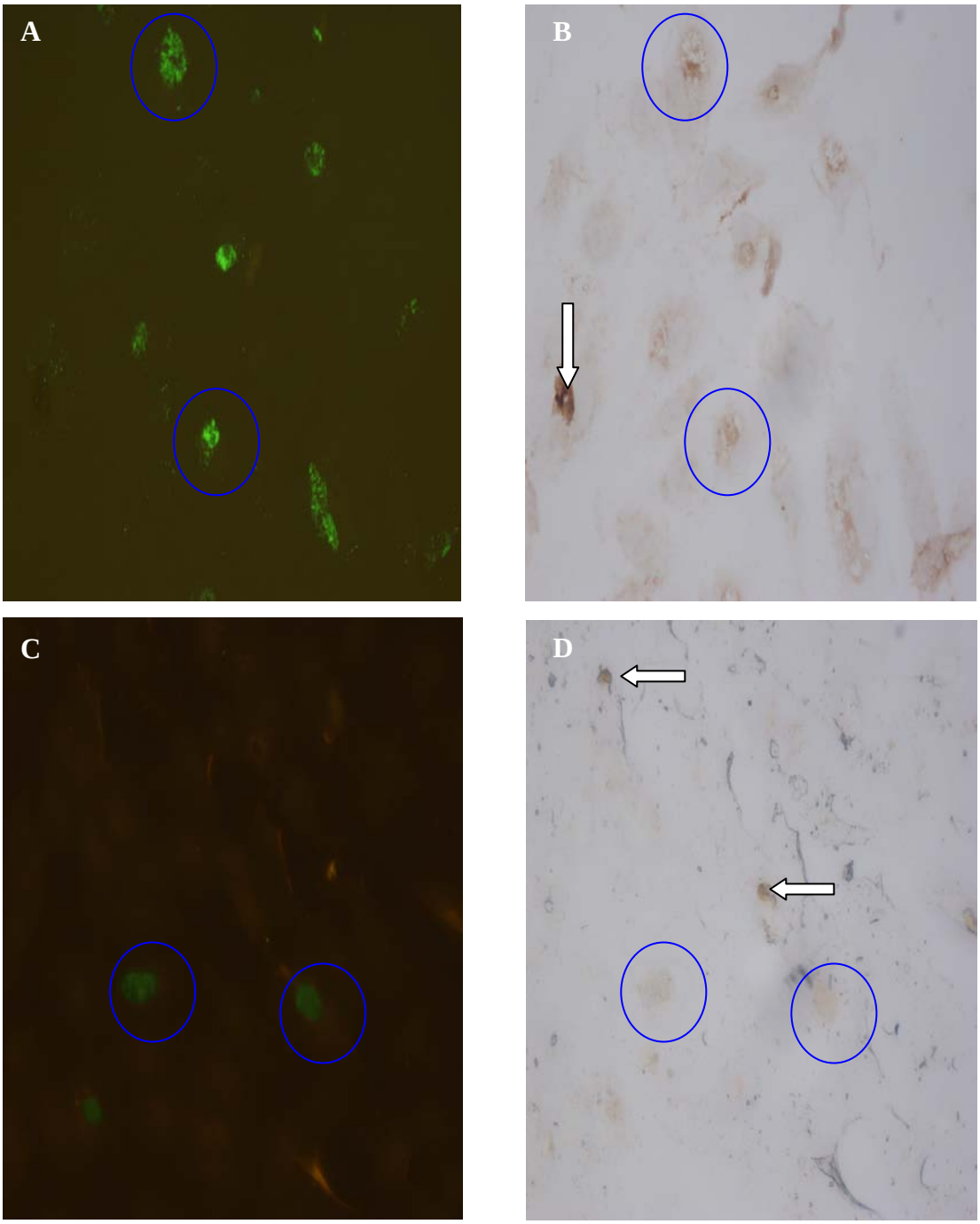


Figure 16: Micrographs of TUNEL stained HUVEC infected with chlamydial serovar L1 (A and B) and L2 (C and D). The micrographs on the left are fluorescent micrographs, the ones on the right are the same field of view bright field TUNEL micrographs. The small dark brown stained nuclei similar to the ones present in the uninfected negative control were present in both the serovars (arrows). The blue circles show the position of inclusion bodies and the corresponding position on the left micrographs, note the light brownish staining, this might show that the DNA was starting to fragment. Original magnification X400.

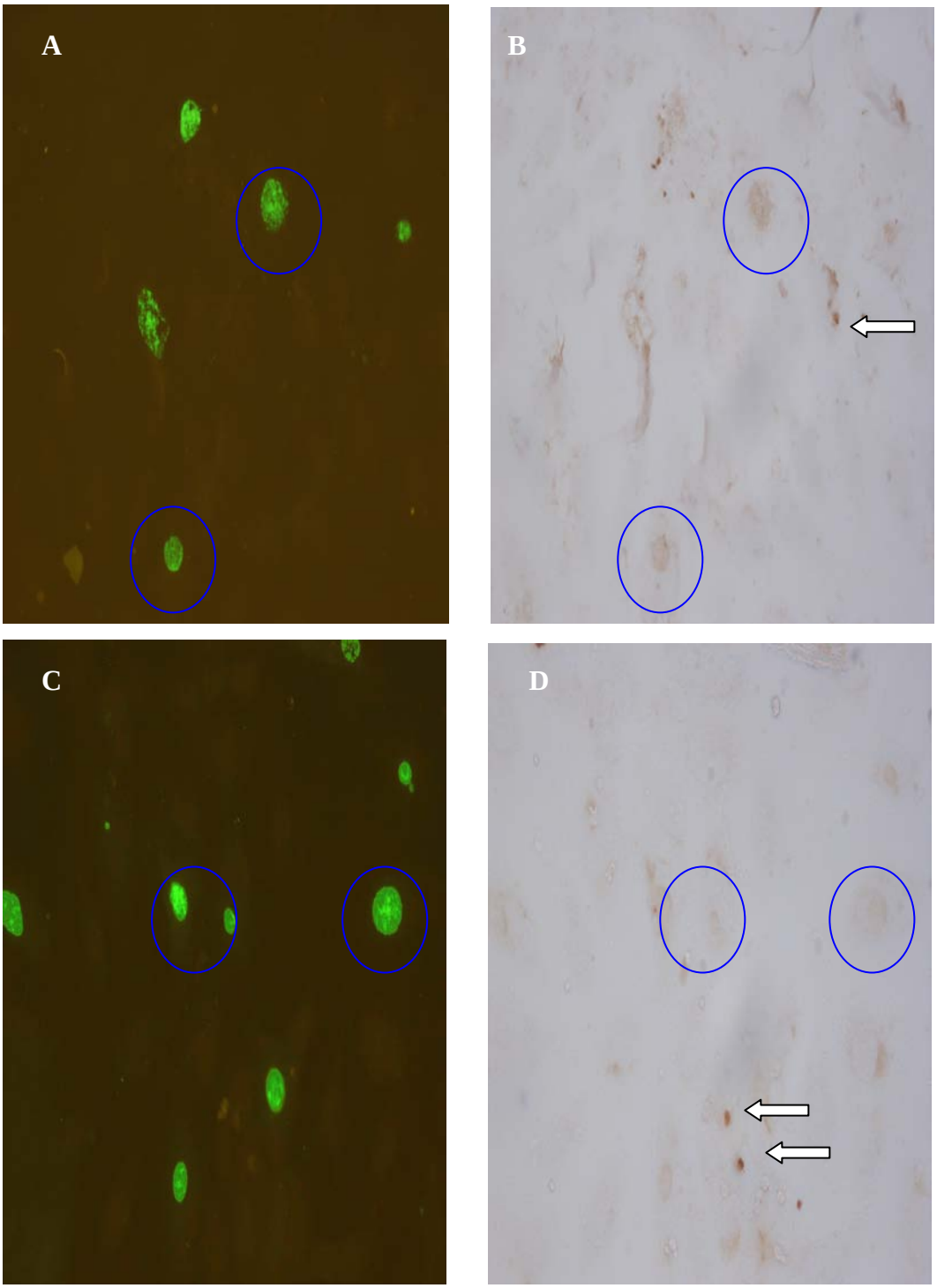
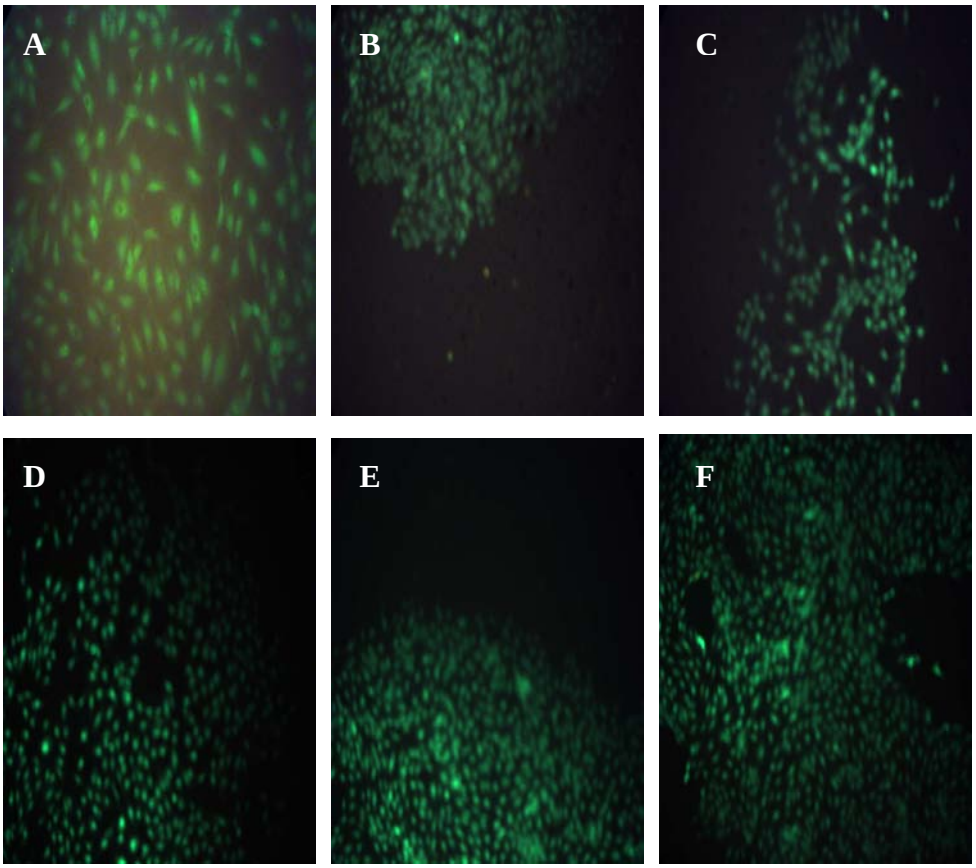


Figure 17: Micrograph of TUNEL stained HUVEC infected with chlamydial serovar L3 (A and B) and E (C and D), 24 hours post exposure. The micrographs on the left are fluorescent micrographs and on the right are the same field of view bright field TUNEL micrographs. The small dark brown stained nuclei is also present here (arrows), and the light brownish staining is also present on the corresponding position of the chlamydial inclusion (blue circle). Original magnification X400.

4.5.2 CaspGLOW^{TM} Flourescein Caspase Staining Assay

Caspase Staining kit was used to confirm cell death by apoptosis after 24 hours of incubation (figure 18). The caspase positive cells in the well treated with etoposide (positive control) were evenly distributed over the coverslip (figure 18A), while the caspase positive cells in the other wells appeared to cluster (figure 18B-G). This clustering and subsequent uneven distribution effect made it difficult to count the number of caspase positive cells. It seemed that there was not much of a difference between caspase positive cells of the controls and chlamydia infected cells and also amongst the serovars. This suggests that death as observed here is due to the normal turnover of the cells.



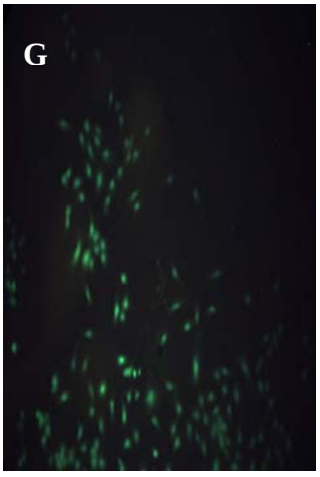


Figure 18: Micrographs of Caspase stained HUVEC. A is positive control (20µM etoposide), notice the evenly distributed caspase positive cells(Original Magnification X200) , B is the negative control, C is the mock infected cells, D-G are L1, L2, L3 and E chlamydial serovars infected cells respectively. There appeared to be no observable difference amongst the serovars, and also between HUVEC infected with chlamydia and the negative and mock infected control. One noticeable feature was that Caspase positive cells appeared in clusters in all experimental wells except for the positive control. Original magnification X100.

CHAPTER 5: DISCUSSION

This study aimed to contribute to the understanding why infection with *C. trachomatis*, biovar LGV results in different pathology as compared with infection with the OG biovar. This was approached by comparing responses of HUVEC to the different LGV serovars with the response to a single OG serovar.

To confirm whether *C. trachomatis* LGV and OG strains do infect and replicate in HUVEC , confluent HUVEC grown in glass slides were infected with *C. trachomatis* L1,L2,L3 and E at an MOI of 1 and stained with MicroTrak® *C. trachomatis* Culture Confirmation Test Kit 24 hours post exposure. Both the LGV and OG *C. trachomatis* serovars successfully infected and replicated in HUVEC, as proven by the presence of inclusion or reticulate bodies which occur after the bacteria has infected the cell as an elementary body transform to a reticulate body which is the replicative phase of the life cycle. This shows that *C. trachomatis* can infect endothelial cells.

Most studies investigate *C. trachomatis* infection of other cell lines such as HeLa and McCoy cell line (Ward *et al*, 1998). However recently, studies have emerged that looked at activation of endothelial cells. Most of these investigate the presence of inclusion bodies after 48 hours and with the use of a cell replication inhibitor like cyclohexamide (Ward *et al*, 1998). We studied the growth of chlamydia without such inhibitors since this reflects what happens *in vivo* more closely. We found intracellular inclusion body-like structures as early as 24 hours after infection (figure 8). Thus *C. trachomatis* is able to attach to and infect HUVEC, get internalized, transforms into reticulate bodies, replicates and form inclusion bodies within 24 hours of exposure. These results correlate with those by Krüll *et al.* (2004) who also showed

that *C. trachomatis* was able to infect and replicate and form inclusion body-like structures in HUVEC 24 hours post exposure. They had a higher percentage of infected cells but this can be attributed to the higher MOI used (MOI=5 versus MOI=1).

Knowing that the bacterium was able to infect HUVEC, the next step was to establish whether it does activate and cause an acute inflammatory response by producing proinflammatory chemokines and increase expression of adhesion molecules leading to leukocyte recruitment and transendothelial migration. The acute inflammatory response to chlamydial infection is characterised by migration of neutrophils to the site of infection followed by monocytes (Stephens, 2003). IL-8 chemokines are specific for recruiting neutrophils whereas MCP-1 chemokines are specific for monocytes. Adhesion molecules are also important in that they act as a binding site for the leukocytes in the blood vessels at the site of infection. Therefore the production of IL-8 and MCP-1 as well as ICAM-1 by HUVEC following exposure with different chlamydia serovars was investigated.

Amongst all LGV strains, L3 was the only serovar which stimulated significantly higher amount of IL-8, MCP-1 and ICAM-1 in HUVEC compared to the negative and mock infected controls. Focusing on *C. pneumoniae,* former studies used L2 as a representative strain for the LGV biovar and hence conclude that all LGV strains do not induce chemokine and adhesion molecule production. They then reach a conclusion that up regulation of these pro-inflammatory factors is unique to *C. pneumoniae*. For instance, a study by Molestina *et al.* in 1999 demonstrated that L2 failed to stimulate production of IL-8 and MCP-1 after 24 hours of incubation

whereas the *C. pneumoniae* strains did. Another study by Krüll *et al*. (2004) also used L2 and showed that after 24 hours of incubation L2 was able to infect HUVEC but did not stimulate any production of IL-8 and MCP-1. Once again *C. pneumoniae* strains were able to induce production of chemokines hence it was concluded that it is an attribute unique to this species.

Our results correlate with the above authors' studies in that L2 did not induce production of IL-8, MCP-1and ICAM-1 after 24 hours of incubation. The same was observed with serovar L1. However unlike other studies we also included L3. There is no literature on effect of L3 on HUVEC in terms of up regulation of proinflammatory factors.

C. trachomatis strains are grouped into biovars based on differences in clinical presentation which correlates with differences of the MOMP and serological differences in OMP-A and sequence differences of the ompA gene (Wang and Grayston, 1974). Attempts to relate the MOMP structure to virulence have been unsuccessful. It has been hypothesised that immune pressure causes MOMP variability hence strain differences but so far there has been no successful studies done to validate this (Byrne, 2010).

Extensive phylogenetic analysis done on the sequence of the *omp*A gene showed some interesting results. It was found that even though L1 and L2 belonged to the same genotype cluster, L3 did not belong to this cluster (Fitch *et al.,* 1993). This might explain the different behaviour observed by L3 in this study. From this follows

the possibility that virulence factors which are responsible for inflammation within 24 hours are produced by L3 which the other two strains do not have.

Virulence factors attributed to *C. trachomatis* virulence include the following: Type III chlamydial secretion proteins responsible for presenting the bacterial effector proteins to the host (Cornelis and Van Gijsegem, 2000), the polyphormic outermenbrane proteins (pmps), which act as autotransporters (Stephens *et al.*, 1998), the putative chlamydial cytotoxin, stress response proteins and the cryptic plasmid (Byrne, 2010). However more work need to be done to relate these factors to strain variation in virulence and classification (Byrne, 2010). Another possibility might be that even though the LGV serovars cause the same damage.i.e. lymphadenitis, maybe they achieve this by means of different pathogenetic mechanisms. L3 might induce inflammation earlier than L1 and L2, and through a different pathway.

Another factor that could explain the observed differences between LGV serovars is loss of virulence during passaging the organisms in in-vitro culture systems. The passage number of the strains we used is unknown but these are old laboratory strains. Since L2 is used in most laboratory studies, it can be assumed that that strain has a higher passage number than the others. The possibility that L3 is the one with the lowest passage number can not be excluded.

The differences between the serovars reported here could relate to differences in pathogenicity. However, no difference in clinical presentation has been observed. Joubert and Sturm (2011) reported on the difference in behaviour between the L2 reference strain and clinical isolates of the same serovar. They conclude that

observations made with reference strains might not be representative for hostcell-chlamydia interaction. This conclusion was reached when the work presented here had been completed. The work will be extended with fresh clinical isolates as a separate project.

After 24 hours of incubation, there was no difference in up regulation of IL-8, MCP-1 and ICAM-1 between the LGV serovar L1-L2 and the OG serovar E. L3 was the only LGV serovar which stimulated the pro-inflammatory proteins. L1 and L2 might behave similarly because they fall phylogenetically in the same ompA sequence cluster. Interestingly, serovar E was grouped with L1 and L2 which might explain why there was no difference between these serovars. This correlates with studies by Molestina *et al.* (1998), who showed that both serovar E and L2 did not induce significant production of IL-8 and MCP-1 after 24 hours of HUVEC infection. However, it must be noted that although no differences were observed between L1, L2 and E after 24 hours, this does not necessarily mean that there would not be any differences with increase in time. It is possible that with increasing time there might be distinct differences between LGV and OG biovars because they have different disease outcome. Further studies should be done based looking at the effect of different serovars on HUVEC with increasing time.

L3 had the highest chemotactic effect on monocytes which correlates with MCP-1 induction experiments. L1, L2 and E did not stimulate any significant migration of monocytes compared to the mock infected cells, which correlates with lack of significant induction of MCP-1 by these serovars. There was no significant stimulation of migration of neutrophils as compared to the negative and mock infected

controls by all the chlamydia serovars. However despite not being significant, L3 has the most positive effect on neutrophil migration and this is in keeping with its stimulation of IL-8 production by HUVEC. Molestina *et al.,* 1998 showed a slight increase in neutrophil migration on exposure to L2 and a significant induction of transendothelial migration of monocytes despite lack of MCP-1 production. Our results confirm these observations.

Serovar E had the least migratory effect for both monocytes and neutrophils; the number of migrated neutrophils was the same as with the mock infected HUVEC. Thus after 24 hours of infection the LGV serovars do stimulate more recruitment and transendothelial migration of monocytes and neutrophils as compared to the OG serovar. This is in keeping with the more severe acute inflammation as seen clinically.

The transendothelial migration assay results could not be tested for normality because they were only three values for each experiment instead of nine. The assay was labour intensive and long and hence could not be done in triplicates in a day by one person without compromising the outcome due to technical errors. Monocytes and neutrophils also had to be isolated from the blood on the same day of experiment and not more than two runs could be done at once. It was therefore decided to do one well per serovar for monocytes and neutrophil transendothelial migration.

The lactate dehydrogenase (LDH) cytotoxicity assay was used to measure cell death due to necrosis of HUVEC infected with different *C.trachomatis* serovars after 24 hours of infection. Host cell lysis has been shown to be a mechanisms used by *C. trachomatis* elementary bodies to leave the infected cell (Todd and Caldwell, 1985).

Moulder *et al.* (1976) have also reported a damaging cytotoxic effect on cells infected with *C. trachomatis* at high MOI (=5). Furthermore, *C. trachomatis* cytotoxicity has been described as early as 1944 by Rake and Jones on macrophages.

L3 was the only serovar which caused significantly higher percentage of cytotoxicity compared to the negative control but not compared with the mock infected cells. The rest of the serovars however were not significantly different from all controls. There was no significant difference between the LGV serovars and OG serovar. L3 might have multiplied more rapidly and started shedding the EBs and hence caused early cell lysis. This needs further investigation. Lack of measurable LDH by other serovars might mean that they might not have reached a point in their life cycle where they had to leave the host cell to infect others. Moulder *et al.* (1976) reported cytotoxicty at a higher MOI (=5). This suggests that indeed, cell lysis is related to the number of chlamydia in the cell.

A cytotoxicity associated gene known as clostridial toxin A gene was identified by Belland *et al*. (2001). These authors reported that L2 has a deletion in the region where this gene is located. They attributed this to the lack of cytotoxicity of L2 in their experiments and reasoned that lack of L2 cytotoxicity on epithelial cells allows it to infect monocytes and disseminate to the lymph nodes. This statement has been quoted by others and has been extrapolated for all LGV serovars (Byrne, 2010).

C. trachomatis has been reported to have apoptotic and anti apoptotic properties. Being an intracellular organism it needs the host cell for survival and thus has developed strategies to keep the host cell viable. On the other hand the organism

needs to exit the cell to infect other cells and therefore has to kill the cell, possibly through inducing apoptosis.

With the TUNEL assay, some small brown apoptotic bodies were observed on the negative control and mock infected controls in all experiments (figure 15). These bodies are smaller in size compared to the DNase treated positive control. They were also seen in uninfected cells in between the infected HUVEC (figure 16 and 17). These apoptotic bodies can be attributed to the normal cell turn over since they were also observed in the negative control. Cells have been shown to die through apoptosis during normal cell development (mitosis and cytokinesis) and tissue homeostasis, inorder to maintain stable cell population in the body (Lockshin and Zakeri, 2004; Steller, 1995). The nucleus of these cells normally shrinks and condenses whereas the membrane still remains intact (Lockshin and Zakeri, 2004; Steller, 1995). The small brown apoptotic bodies observed in our setting are typical of these fragments.

Analysis of the infected cell by counterstaining TUNEL staining with *C. trachomatis* staining test revealed no apoptotic positive nuclei in the infected cells. This was common in all the LGV and OG *C. trachomatis* infected cells. However, where the infected cell was positioned, a faint brown colour was observed. This might mean that the infected cell was in a process of dying and the nucleus was starting to fragment. It has been shown that cells infected with chlamydia resist apoptosis until they have completed their intracellular cycle prolonging the life of the cell they inhabit (Dean and Powers, 2001). This anti-apoptotic activity is attributed to the up regulation of the *bcl*-2 gene by *C. trachomatis* during the reticulate body stage thus inhibiting apoptosis during the first 24 hours of infection (Dean and Powers, 2001). In this study cells

were only incubated for 24 hours hence the anti-apoptotic systems were still in place. Apoptosis of infected cell might have occurred with increase in incubation time as the reticulate bodies transform into elementary bodies hence down regulation of the *bcl*-2 gene.

The TUNEL assay has been criticised for not being able to discriminate between necrotic cells which have gone through DNA laddering and apoptotic cells (Grasl-Kraupp *et al.,* 1995; de Torres *et al.,* 1997). This assay was chosen for this study because it made it able for the infected cells to also be counterstained with the *C. trachomatis* confirmation stain to provide evidence that the cells were indeed infected.

To further confirm whether cell death on chlamydia infected HUVEC was by apoptosis or some other mechanism, the CaspGlow Flurescein Caspase Staining assay was performed. This assay is thought to be specific in that it binds to activated capsases. There seemed to be no difference between the uninfected negative control, mock infected control and LGV and OG serovars infected HUVEC. This supports what was observed with the TUNEL assay.

The disadvantage of this method was that it did show apoptotic cells clusters in one part of the slide and not in others. Hence counting of stained cells in different field of view could not be carried out. This might have been due by the observation that conjugating caspase inhibitors with peptide markers which mimic the caspase cleavage sites leads to non-specific inhibition due to overlapping of caspase activity since this assay also has an inhibitor (VAD-FMK) bound to a marker (FITC) (Thornberry *et al.*, 1997; Villa *et al.*, 1997). This assay measures the enzyme activity in a population of cells and not in a single cell (Vermes *et al.,* 2000). The authors

suggest that flow cytometry can be used interrogate individual cells which was not employed in our setting.

It must be noted that some other types of postulated cell death mechanisms such pyroptosis, autophagy and oncosis may be involved. However, there are no established laboratory techniques as yet to measure them. Some researchers have stipulated that cell death may involve numerous pathways and communication between the pathways allows for a controlled form of death. They postulate that no distinct cell death programme is responsible for the death of a cell, but that there is an overlap of several cell death pathways (Ankarcrona *et al.*, 1995; Kostin *et al.*, 2003; Fink and Cookson., 2005).

CHAPTER 6: CONCLUSION

Our results show that all the LGV and OG *C.trachomatis* serovars are able to infect and replicate in HUVEC. However only L3 was able to stimulate significant levels of proinflammatory factors (IL-8, MCP-1 and ICAM-1) and stimulated migration of the highest number of monocytes and neutrophils compared to other serovars after 24 hours of incubation. Comparison of LGV and OG serovars showed no significant difference between these two biovars in inducing production of IL-8 and MCP-1, but L3 stimulation of ICAM-1 was significantly higher than E. Cell death by necrosis was only significant in cells infected by L3 serovar. Serovar L3 seems to stimulate a more rapid acute immune response compared to the other serovars. The rest of the LGV serovars do not differ from the OG serovar E in inducing inflammation in the first 24 hours of infection.There was no evidence of cell death by apoptosis due to any chlamydial serovar supporting studies by Gupta, 2001 that chlamydia activates the anti-apoptotic Bcl-2 gene to inhibit apoptosis of the infected cell so that it can complete its intracellular life cycle. Further studies need to be done to compare these serovars activation and infect in/ on HUVEC at different time intervals (increasing time). Studies with fresh clinical isolates need to follow to rule out the effect of multiple passages in vitro.

REFERENCES

Alexander S., Martin, I. M. C and Ison, C**.** 2008. A comparison of two methods for the diagnosis of lymphogranuloma venereum. *Journal of Medical Microbiology*, 57: 962-965.

Al-Numani S. M., Dore, M. and Gottschalk, M.2003. Up-regulation of ICAM-1, CD11a/CD18 and CD11c/CD18 on human THP-1 monocytes stimulated by *Streptococccus suis* serotype 2. *Clinical and Experimental Immunology*, 133: 67-77.

Ankarcrona, M., Dybbukt, J. M. E., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton, S.A and Nicotera P. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron,* 15: 961-973.

Barnes, R.C. 1989. Laboratory diagnosis of Human Chlamydial infections. *Clinical Microbiology Reviews,* 2:119-136

Bella J., Kolatkar P.R., Marlor C.W., Greve J.M. and M.G. Rossmann M.G. 1998. The structure of the two amino-terminal domains of human ICAM-1 suggests how it functions as a rhinovirus receptor and as an LFA-1 integrin ligand .*Proc Natl.Acad. Sci*. USA. 95(8): 4140-4145

Belland. R. J., Scidmore, M.A., Crane, D.D., Hogan D.M., Whitmire, W., McClarty G. and Caldwell, H.D. 2001 *Chlamydia trachomatis* cytotoxicity associated with complete and partial cytotoxin genes. *PNAS,* 98: 13984-13989

Blagosklonny, M. V. 2000. Cell death beyond apoptosis. *Leukemia,* 14**:**1502-1508

Boatright, K. M. and Salvesen, G.S**.** 2003. Mechanisms of caspase activation. *Curr. Opin. Cell Biol.*, 15:725-731.

Brackenbury R., Rutishauser, U. and Edelman G.M. 1981. "Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells". *Proc. Natl. Acad. Sci. U.S.A.* 78 (1): 387–91

Bursch, W., Ellinger, A., Gerner, C., Frohwein, U. and Schulte-Hermann R**.** 2000. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others*? Ann. N. Y. Acad. Sci.* 926**:**1-12.

Bursch W. 2001. The autophagosomal-lysososomal compartment in programmed cell death. *Cell Death Differ*. 8:569-581.

Byrne G.I. 2010. *Chlamydia trachomatis* Strains and Virulence: Rethinking Links to Infection Prevalence and Disease Severity. *Journal of Infectious Diseases,* 201(S2): S126-S133.

Centers for Disease Control and Prevention. 2006. Sexually Transmitted Diseases Treatment Guidelines . Morbidity and Mortality Weekly Report Department of Health and Human Services, Atlanta, USA, 55 **:**(No. RR-11).

Chernesky, M.A., Mahony, S. and Castriciano, S**.** 1986. Detection of Chlamydia trachomatis antigens by enzyme immunoassay and immunofluorescence in genital

specimens from symptomatic and asymptomatic men and women. *J. Infect Dis.*, 154:141-8

Chernesky M.A. 2005. The laboratory diagnosis of *Chlamydia trachomatis* infections *J Infect Dis. Med Microbiol.,* 16(1): 39–44.

Clarke, P. G. 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol. (Berlin) 181**:**195-213.

Clarke L.M., Sierra M.F., Daidone, B.J., Lopez, N., Covino, J.M. and McCormack W.M. 1993. Comparison of the Syva MicroTrak enzyme immunoassay and Gen-Probe PACE 2 with cell culture for diagnosis of cervical *Chlamydia trachomatis* infection in a high-prevalence female population. *J Clin Microbiol*., 31:968-971.

Collins, R. J., Harmon, B.V., Globe, G.C., and Kerr, J.F. 1992. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Radiat. Biol*., 61:451-453.

Cornelis, G.R. and F. Van Gijsegem .2000. Assembly and function of type III secretion systems. *Annu Rev Microbiol*., 54:735–774.

Creagh, E. M., Conroy, H., and Martin S.J. 2003. Caspase-activation pathways in apoptosis and immunity. *Immunol Rev*., 193:10-21.

Dacheux, D., Toussaint B., Richard, M., Brochier, G., Croize, J., and Attree I. 2000. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infect. Immun.*, 68(5):2916-2924.

Dean, D. and Powers, V.G. 2001. Persistent *Chlamydia trachomatis* infections resist apoptotic stimuli. *Infection and Immunity*, 69: 2442-2447.

de Torres, C., Munell,F., Ferrer, I., Reventos,J., and Macaya, A. 1997. Identification of necrotic cell death by the TUNEL assay in the hypoxic-ischemic neonatal rat brain. *Neurosci. Lett*., 230:1-4.

Dong, F., Pirbhai M., Xiao Y., Zhong Y., Wu, Y., and Zhong, G**.** 2005. Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in *Chlamydia trachomatis*-infected cells. *Infect. Immun*., 73(3):1861-1864.

Engelhardt, B. and Wolburg, H. 2004. Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol.*, 34:2955–2963.

Everett, K.D.E., Bush, R.M. and Andersen, A.A. 1999.Emended description of the order *Chlamydiales*: proposal of *Sarachlamydiace* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards *International Journal of Systematic Bacteriology*, 49: 415-440

Fajardo, L.F.1989. The complexity of endothelial cells.*Am.J.Clin.Path*., 92: 241-250

Fan T., Lu, H., Hu, H., Shi, L., McClarty G. A., Nance, D. M., Greenberg, A .H. and Zhong G. 1998. Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med.,* 187:487–496

Fernandez, E. and Lolis E**.** 2002. "Structure, function, and inhibition of chemokines". *Annu Rev Pharmacol Toxicol*., 42: 469–99

Fischer, S. F., Harlander, T., Vier, J and Hacker G**.** 2004. Protection against CD95-induced apoptosis by chlamydial infection at a mitochondrial step. *Infect. Immun*., 72:1107-1115.

Fischer, S. F., Vier, J., Kirschnek, S., Klos, A., Hess, S., Ying, S. and Hacker G. 2004. *Chlamydia* inhibit host cell apoptosis by degradation of proapoptotic BH3-only proteins. *J. Exp. Med.,* 200:905-916

Fitch, W, M., Peterson E.M and de la Maza L.M. 1993. Phylogenetic analysis of the outer membrane protein genes of *Chlamydia* and its implication for vaccine development. *Mol Biol Evol.,* 10:892–913.

Fink, S.L. and Cookson B.T. 2005. Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infection and Immunity,* 73(4): 1907-1916.

Fukuda E.T., Lad, S.P., Mikolon, D.P., Iacobelli-Martinez, M., and Erguang Li. 2005. Activation of Lipid Metabolism Contributes to Interleukin-8 Production during *Chlamydia trachomatis* Infection of Cervical Epithelial Cells. *Infection and Immunity*, 73: 4017-4024.

Gibellini, D., Panaya, R. and Rumpianesi, F**.** 1998. Induction of apoptosis by *Chlamydia psittaci* and *Chlamydia trachomatis* infection in tissue culture cells. *Zentbl. Bakteriol.,* 288:35-43.

Gift, T.L., Pate, M.S., Hook, E.W., and Kassler, W.J. 1999. The rapid test paradox: When fewer cases detected lead to more cases treated. *Sex Transm Dis*., 26: 232-240.

Gomes, J.P., Bruno, W.J., Borrego, M.J. and Dean, D. 2004. Recombination in the genome of *Chlamydia trachomatis* involving the polymorphic membrane protein C gene relative to ompA and evidence for horizontal gene transfer. *Journal of Bacteriology*, 186(13): 4295-4306.

Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska,K., Bursch,W. and Schulte-Hermann R. 1995. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology* , 21(5):1465-1468.

Grayston, S., Kuo, C.C., Wang, S.P and Altman, J.1986. A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med*., 315: 161-168

Greenwood J., Amos, C.L., Walters, C.E., Couraud, P.O., Lyck, R., Engelhardt, B. and Adamson P. 2003. Intracellular domain of brain endothelial intercellular adhesion molecule-1 is essential for T lymphocyte-mediated signalling and migration. *J Immunol.*, 171(4):2099–2108.

Gupta S. 2001. Molecular steps of death receptor and mitochondrial pathways of apoptosis. *Life Sciences***,** 69(25)**:** 2957–2964.

Hasegawa, M., Sato, S. and Takehara, K. 1999. Augmented production of chemokines (monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β) in patients with systemic sclerosis: MCP-1 and MIP-1α may be involved in the development of pulmonary fibrosis. *Clin Exp. Immunology*, 117(1): 159-165

Hedgecock, E. M., Sulston, J.E. and Thompson**,** J.N.1983. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science*, 220**:**1277-1279.

Hersh, D., Monack, D.M., Smith,M.R., Ghori, N., Falkow, S. and Zychlinsky, A. 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci., USA* 96:2396-2401.

Hirano, T. and Ruebner, B.H. 1966. Studies on the mechanism of destruction of lymphoid tissue in murine hepatitis virus (MHV3) infection. I. Selective prevention of lymphoid necrosis by cortisone and puromycin. *Lab. Investig.*, 15:270-282.

Hsia, R.C., Pannekoek, Y., Ingerowski, E., Bavoil, P.M.1997. Type III secretion genes identify a putative virulence locus of *Chlamydia. Mol. Microbiol.*, 25:351–359.

Jean-Luc, P., Darville, T., Gachelin, G., Souque, P., Huerre, M., Dauty-Varsat, A., and Ojcius, D.M. 1999. Effect of *Chlamydia trachomatis* and Subsequent Tumor Necrosis Factor Alpha Secretion on Apoptosis in the Murine genital tract. *Infection and Immunity*, 68: 2237-2244

Joubert, B. 2009. The interaction of lymhogranuloma venereum and oculogenital *Chlamydia trachomatis* with human keratinocytes and cervical epithelium, PhD Thesis, University of Kwa-Zulu Natal.

Kerr, J. F., Wyllie, A.H. and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, 26:239-257.

Kostin, S., Pool, L., Elsasser, A., Hein, S., Drexler, H.C., Arnon, E., Hayakawa, Y., Zimmermann, R., Bauer, E., Klovekorn, W.P. and Schaper, J. 2003. Myocytes die by multiple mechanisms in failing human hearts. *Circ. Res.*, 92:715-724.

Krüll, M., Nost, R., Hippestiel, S., Domann, E., Chakraborty, T.J. and Suttorp.N. 1997. *Listeria monocyogenes* potentially induces up-regulation of endothelial adhesion molecules and neutrophil adhesion to cultured human endothelial cells. *Journal of Immunology,* 159:1970-1976.

Krüll, M., Klucken, A.C., Wuppermann, F.N., Fuhrmann, O., Seybold, J., Hippenstiel, S., Hegemann, J.H., Jantos, C.A. and Suttorp.N. 1999. Signal transduction pathways activated in endothelial cells following infection with Chlamydia pneumoniae. *Journal of Immunology,* 162:4834-4841.

Krüll, M., Kramp, J., Petrov, T., Klucken, A. C. Hocke, A.C. Walter, C., Schmeck, B., Seybold, J., Maass, M., Ludwig, S., Kuipers, J.G., Suttorp, N. and Hippenstiel' S. 2004. Differences in Cell Activation by *Chlamydophila pneumoniae* and *Chlamydia trachomatis* Infection in Human Endothelial Cells. *Infection and Immunity*, 72(11): 6615-6621.

Lecoeur, H., Prevost, M.C. and Gougeon, M.L. 2001. Oncosis is associated with exposure of phosphatidylserine residues on the outside layer of the plasma membrane: a reconsideration of the specificity of the annexin V/propidium iodide assay. *Cytometry,* 44:65-72.

Levin, S. 1998. Apoptosis, necrosis, or oncosis: what is your diagnosis? A report from the Cell Death Nomenclature Committee of the Society of Toxicologic Pathologists. *Toxicol.Sci.*, 41:155-156.

Levin, S., Bucci, T.J., Cohen, S.M., Fix A.S., Hardisty, J.F., LeGrand, E.K., Maronpot, R.R, and Trump, B.F. 1999. The nomenclature of cell death: recommendations of an ad hoc committee of the Society of Toxicologic Pathologists. *Toxicol. Pathol.* 27:484-490.

Lockshin, R. A. and Zakeri, Z. 2004. Apoptosis, autophagy, and more. *Int. J. Biochem. Cell Biol*., 36:2405–2419.

Lowman, H.B., Slagle, P.H., DeForge, L.E., Wirth, C.M., Gillece-Castro, B.L., J. H. Bourell, J.H. and Fairbrother, W.J. 1996. Exchanging Interleukin-8 and Melanoma Growth-stimulating Activity Receptor Binding Specificities. *The Journal of Biological Chemistry*, 271:14344-14352

Lyck, R., Reiss, Y., Gerwin, N., Greenwood, J., Adamson, P., and Engelhardt, B. 2003. T-cell interaction with ICAM-1/ICAM-2 double-deficient brain endothelium in vitro: The cytoplasmic tail of endothelial ICAM-1 is necessary for transendothelial migration of T cells. *Blood*, 102(10):3675–*3683.*

Mabey, D. and Peeling, R.W. 2002. Lymphogranuloma venereum. *Sex. Transm. Infect*., 78; 90-92.

Majno G and Joris. I. 1995. Apoptosis, oncosis, and necrosis: an overview of cell death. *Am J Pathol*., 146:3–15.

Maleka, D.M, Hoosen, A.A., Sturm, A.W. and Kiepiela, P. 1996. The Laboratory Diagnosis of Lymphogranuloma venereum (LGV). MMedSci. dissertation, University of Natal MedicalSchool, Department of Medical Microbiology, Durban, South Africa. (unpublished).

Mariotti, S.P. 2004. "New steps toward eliminating blinding trachoma". *N. Engl. J. Med.*, 351(19): 2004–2007.

McGeachie, J. 1998. Blue histology-vascular system, more about endothelial cells, School of Anatomy and Human Biology, University of Western Australia.

Meijer, C.J.L.M. and Van den Brule, J.C. Urogenital Chlamydia trachomatis Serovars in Men and Women with symptomatic or an asymptomatic Infection: an Association with Clinical Manifestations. *Journal of Clinical Microbiology*, 38: 2292-2296.

Molestina, R. E., Dean, D., Miller, R.D., Ramirez,J.A. and Summersgill, J.T. 1998 Characterization of a Strain of Chlamydia pneumoniae Isolated from a Coronary Atheroma by Analysis of the omp1 Gene and Biological Activity in Human Endothelial Cells. *Infection and Immunity*, 66(4): 1370-1376.

Molestina, R.E., Miller, R.D., Ramirez, J.A. and Summersgill, S. 1999. Infection of Human Endothelial Cells with *Chlamydia pneumoniae* Stimulates Transendothelial Migration of Neutrophils and Monocytes. *Infection and Immunity*, 67: 1323-1330.

Moravec, R. 1994. Total cell quantification using the CytoToxTM Assay Non-Radioactive Cytotoxicity Assay. *Promega Notes* 45: 11-12.

Morre' S.A., Rozendaal A.L., Van Valkengoed, G.M, Boeke, A.J.P., Van Voorstvader, P.C., Schirm, J., de Block, S., Van der Hoek,A.R., van Doornum, J.J., Moulder, J. W., Hatch, T.P., Byrne, G.I., and K. R. Kellogg. 1976. Immediate toxicity of high multiplicities of *Chlamydia psittaci* for mouse fibroblasts (L cells). *Infect. Immun.* 14:277-289.

Moulder, J.W. 1991. Interaction of Chlamydiae and Host Cells in vitro.55: 143-190.

Nisato, R. E, Harrison, J.A., Buser, R., Orci, L., Rinsch, C., Montesano, R., P. Dupraz, P. and Pepper.M.S. 2004. Generation and Characterization of Telomerase-Transfected Human Lymphatic Endothelial Cells with an External Life Span. Technical Advance. *American Journal of Pathology*, 165(1):11-24

Obregon, C., Dreher, D., Kok, M., Cochand, L., Kiama, G.S. and Nicod, L.P. 2003. Human alveolar macrophages infected by virulent bacteria expressing SipB are a major source of active interleukin-18. *Infect. Immun.*, 71:4382-4388.

Ojcius, D. M., Souque, P., Perfettini, J.L. and Dautry-Varsat, A. 1998. Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen *Chlamydia psittaci*. *J. Immunol.*, 161:4220-4226

Rahman, A. and Fazal, F. 2009. Hug tightly and say goodbye: Role of endothelial ICAM-1 in leukocyte transmigration. Antioxidants and redox signalling 11(4): 823-839.

Rake, G and Jones, H.P. 1944. Elementary Body Envelopes from Chlamydia psittaci Can Induce Immediate Cytotoxicity in Resident Mouse Macrophages and L-Cells. *J Exp Med.*, 79: 463–485.

Reacher, M., Foster, A. and Huber,J. 1993. "Trichiasis Surgery for Trachoma. The Bilamellar Tarsal Rotation Procedure." World Health Organization, WHO/PBL/93.29, Geneva.

Samali, A., Zhivotovsky, B., Jones, D., Nagata, S. and Orrenius, S. 1999. Apoptosis: cell death defined by caspase activation. *Cell Death Differ.*, 6:495-496

Samuel, R.G., and Stephens, R.S. 2001. Rapid, transient phosphatidylserine externalisation induced in host cells by infection with *Chlamydia* spp. *Infection and Immunity*, 69(2): 1109-1119

Schoier J., Hogdahl, M., Soderlund, M. and Kihlstrom, E. 2006. *Chlamydia (Chlamydophila) pneumoniae*-induced cell death in human coronary artery endothelial cells is caspase-independent and accompanied by subcellular translocations of Bax and apoptosis-inducing factor. *Immunology & Medical Microbiology*, 47: 207-216.

Simon, H.U.I., Tsao, P.W., Siminovitch, K.A., Mills, G.B. and Blaser, K. 1994. Functional platelet-activating factor receptors are expressed by monocytes and granulocytes but not by resting or activated T and B lymphocytes from normal individuals or patients with asthma. *J Immunol.*, 153: 364–377.

Skelton N.J, Quan, C., D. Reilly, D. and Lowman, H. 1999. Structure of a CXC chemokine-receptor fragment in complex with interleukin-8. *Structure*, 7:157-168.

Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature*.346:425–434.

Staunton D.E., Marlin, S.D., Stratowa, C., Dustin, M.L.and Springer, T.A. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell*, 52:925–933.

Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science*, 267: 1445-1449

Stenner-Liewen, F., Liewen, H., Zapata, J.M., Pawlowski, K., Godzik, A. and Reed, J.C. 2002. CADD, a *Chlamydia* protein that interacts with death receptors. *J. Biol. Chem.*, 277:9633-9636.

Stephens, R.S., Kalman, S., Lammel, C, Fan,J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., and Davis R.W. 1998. Genomic sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science*, 282(5389)**:**754–759.

Stephens, R. S. 2003. The cellular paradigm of chlamydial pathogenesis. *Trends Microbiol.*, 11:44-51.

Sturm, P.D., Moodley, P., Khan, N., Ebrahim, S., Govender, K., Connolly C and Sturm, A.W. 2004. Aetiology of male urethritis in patients recruited from a population with a high HIV prevalence. *Int J Antimicrob Agents*. 24 (Suppl 1):S8-14.

Sturm P.D.J., Moodley, P., Govender, K., Bohlken, L., Vanmali, T., and Sturm, A.W. 2005. Molecular Diagnosis of Lymphogranuloma Venereum in Patients with Genital Ulcer Disease. *Journal of Clinical Microbiology*, 43:2973-2975

Sompayrac, L. 2008. How the Immune System Works (3rd ed.). Malden, MA: Blackwell Publishing.

Thornberry, N. A., Rano,T.A., Peterson,E.P., Rasper, D.M.,Timkey,T., Garcia-Calvo, M.,Houtzager,V.W., Nordstrom,P.A., Roy, S.,Vaillancourt,J.P., Chapman,K.T. and Nicholson,D.W. 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.*, 272(29):17907-17911.

Todd, W.J., and Caldwell, H.D. 1985. The interaction of *Chlamydia trachomatis* with hostcells: ultrastructural studies of the mechanism of release of a biovar II strain from HeLa229 cells. *J Infect Dis.*, 151:1037-44.

Utgaard, J.O, Johnson, F.L., Bakka,A., Per Brandtzaeg, and Haraldsen,G**.** 1998. Rapid Secretion of Prestored Interleukin 8 from Weibel-Palade Bodies of Microvascular Endothelial Cells .*J.Exp.Med.,* 188: 1751-1756

Villa, P., Kaufmann, S.H. and Earnshaw. W.C. 1997. Caspases and caspase inhibitors. *Trends Biochem., Sci.,* 22**:**388-393.

Walisser, J. A., and Thies, R.L. 1999. Poly (ADP-ribose) polymerase inhibition in oxidant-stressed endothelial cells prevents oncosis and permits caspase activation and apoptosis. *Exp. Cell Res.,* 251:401-413.

Wang, S.P., and Grayston, S. 1974. Human serology in *Chlamydia trachomatis* infection with microimmunofluorescence. *J Infect Dis*; 130:388-397.

Wang, Q. and Doerschuk,C.M. 2002. The signalling pathway induced by neutrophil-endothelial cell adhesion. *Antioxidants and Redox Signalling*, 4(1): 39-47.

Ward, M. E. 1999. Mechanisms of *Chlamydia*-induced disease, p. 171-210. *In* R. S. Stephens (ed.), *Chlamydia*: intracellular biology, pathogenesis, and immunity. ASM Press, Washington, D.C.

Ward, M.E. and Ridgway, G. 1998: “Chlamydia”, Chapter 59: 1331 – 1346 In: Collier L, Balows A and Sussman M (Editors): Topley and Wilson’s Microbiology and Microbial Infections, Ninth Edition,Volume 2, Publisher: Arnold, London.

Vermes, I., Haanen, C. and Reutelingsperger, C**.** 2000. Flow cytometry of apoptotic cell death*. J. Immunol. Methods* , 243**:**167–190.

WHO, Sexually Transmitted Diseases. 2010. Initiative for Vaccine Research (IVR) 44

Ying, S., Seiffert, B.M., Hacker, G. and Fischer, S.F. 2005. Broad degradation of proapoptotic proteins with the conserved Bcl-2 homology domain 3 during infection with *Chlamydia trachomatis*. *Infect. Immun.*, 73(3):1399-1403.

Zhang Y.J., Rutledge, B.R. and Rollins, B.J. 1994. MCP-1: Structure/Activity Analysis of Human Monocyte Chemoattractant Protein-1(MCP-1) by Mutagenesis. *The Journal of Biological Chemistry*, 269(22): 15918-15924.

Zhang Y.J., Ernst, C A. and Rollins, B.J. 1996. MCP-1: Structure/Activity Analysis. *Methods*: *A Companion to Methods in Enzymology*, 10: 93-103.

Zhang, X.**,** Majlessi**,** L., Deriaud, E., Leclerc**,** C. and Lo-Man, R. 2009**.** Coactivation of Syk Kinase and MyD88 Adaptor Protein Pathways by Bacteria Promotes Regulatory Properties of Neutrophils. *Immunity*, 31(5): 761-771.

Zimmerman, G.A. 1992. Endothelial cell interactions with granulocytes: tethering and signaling molecules*. Immunol Today,* 13:93–100.

Zychlinsky, A., Fitting, C., Cavaillon, J.M., and Sansonetti, J. 1994. Interleukin 1 is released by murine macrophages during apoptosis induced by *Shigella flexneri*. *J. Clin. Investig***.,** 94:1328-1332.

APPENDIX A – Tissue culture reagents and media

McCoy freezing Fluid

Per 100 ml:

30 ml EMEM

10 ml FBS

10 ml DMSO (Fluka, Steinheim, Germany)

To a 100 ml autoclaved bottle add 30 ml of EMEM, 10 ml of FCS and lastly 10 ml of DMSO and swirl gently to mix. Filter sterilise the freezing fluid. Aliquot the freezing fluid onto 10 ml tubes, seal tightly and store in a -20 °C freezer. This must be done under sterile conditions in a safety cabinet. Volumes of the reagents can be altered according to how much of the freezing fluid is needed.

Chlamydia Growth Media/ Inoculation Media

10% FBS

2mM L-Glutamine

1% Non-essential amino acids

4mg of glucose per ml

10mM HEPES

10µg of gentamicin per ml

10µg of cycloheximide per ml

10µg of fungizone per ml

Add all the above reagents to EMEM with Non essential amino acids (NEAA), Earle's balanced salt solution (EBSS) to yield their stated concentrations.

HUVEC freezing fluid

Per 40ml:

30 ml of reconstituted EGM-2 media

5 ml FBS

5 ml of DMSO

Add 5 ml of FBS and 5 ml of DMSO to the EGM-2 media. Filter sterile the fluid through a 0.22 μm filter, aliquot and store at -4°C until required.

Phosphate Buffered Saline (PBS) solution

5 PBS tablets (Oxoid)

500ml distilled water

Add the 5 PBS tablets to a 500 ml sterile bottle containing 500 ml of autoclaved triple distilled water. Allow the tablets to dissolve and then sterilize the solution by autoclaving at 121°C for 10 minutes. Aliquot into 20ml tubes and store in a -20°C freezer until use.

Sucrose-Phosphate-Glutamate Buffer (SPG) buffer

0.260g KH_2PO_4

0.7645g $Na_2HPO_4.7H_2O$

37.5g sucrose

0.360g glutamic acid

500 ml distilled water (500ml)

Dissolve the above reagents in 400 ml distilled water. Adjust the pH of the buffer to 7.5 using the 7.5% sodium bicarbonate. After reaching the target pH adjust the volume of the buffer to 500ml using the remaining distilled water.

APPENDIX B – Objective lens Conversion factors

These are conversion factors for the objective lens of the fluorescence microscope used to determine the concentration of the stored bacterium inoculum. To calculate this, the objective lens area is needed.

The objective lens conversion factors were calculated by dividing the area of the well with the particular objective field area**.**

Table 3: Conversion factors for the objective lens of the fluorescence microscope

Objective magnification	Conversion factor
10X	100
2OX	400
40X	1 600
100X	10 000

APPENDIX C - Preparation of Reagents for ELISA

IL-8 ELISA Reagents

Wash buffer

Pipette 2.5 ml of Wash buffer concentrate (200X) into a clean 500 ml graduated cylinder. Bring the final volume to 500 ml with triple distilled water.

Mix gently to avoid foaming.

Standard diluent buffer

Empty the content of the vial in a 500 ml autoclaved clean bottle containing 225 ml distilled water.

IL-8 Standards

Reconstitute the standard by adding the volume of standard diluent buffer stated on the label of the standard vial.

Biotinylated anti-IL-8

Dilute 120 µl of biotinylated antibody concentrate with 3, 180 ml of biotinylated Antibody diluent in a clean glass vial.

Streptavidin-HRP

This must be prepared just before use and cannot be kept for further experiments. Add 0.5 ml of HRP diluent to the vial containing Streptavidin-HRP to make a 1:100

dilution. Make a further dilution with HRP-diluent in a clean glass vial as needed in this case mix 75 µl of pre-diluted Streptavidin-HRP with 5 ml of HRP diluent

MCP-1 ELISA Reagents

All the reagents were prepared before starting the assay in exception of the HRP-Conjugate.

Wash buffer

Add 25 ml of wash buffer concentrate with into a clean 500 ml graduated cylinder and bring the final volume to 500 ml with triple distilled water. Gently mix to avoid foaming. Measure the pH to confirm that its 7.4.

Assay buffer

Shake the Assay buffer concentrate gently to ensure proper mixture of the contents Pipette 2.5 ml of the assay buffer concentrate to 47.5 ml of triple distilled water. Gently mix to avoid foaming

MCP-1 Standards

Reconstitute the lyophilized standard with distilled water as stated on the standard vial label. Mix gently until the contents have dissolved.

HRP-Conjugate

Mix 0.03 ml of HRP-Conjugate with 2.97 ml of Assay buffer in a clean plastic test tube to make 1:100 dilution.

Addition of Colour-giving reagents: Blue Dye, Green Dye

Before sample dilution add 200 µl of Blue dye to 50 ml of assay buffer (1:250 dilution). Before dilution of the concentrated conjugate add 30 µl of the Green-Dye to 3 ml of the assay buffer used for the final conjugate dilution (1:100 dilution).

ICAM ELISA Reagents

1% formaldehyde

Add 17 ml of PBS (pH 7.4, Oxoid) to a 20ml tube and then add 0.5ml of 35% formaldehyde. Swirl gently to mix.

50µg/ml anti-ICAM

Reconstitute the 500µg anti-humanICAM-1 purified mouse monoclonal IgG_1 to a stock concentration of 0.5mg/ml by adding 1ml of sterile PBS (pH 7.4, Oxoid). Store in a -20°C until further use .Before starting the assay get the reconstituted anti-hICAM from the freezer and allow it to come to room temperature. To 5 ml of sterile PBS (pH 7.4, Oxoid) in a 15 ml tube add 0.5µl of the reconstituted anti-ICAM. Swirl gently to mix.

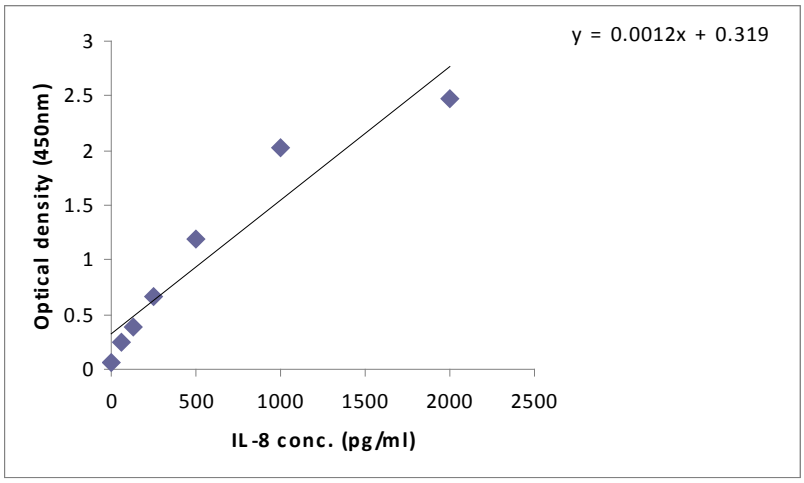
APPENDIX D - Raw data from experiments

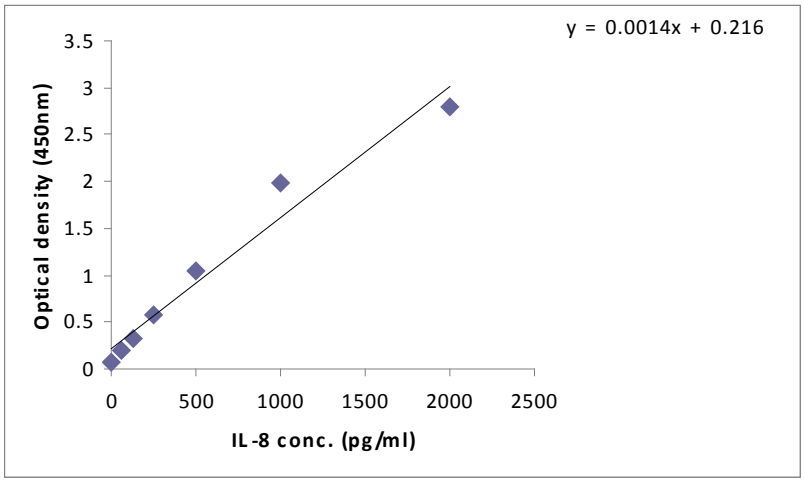
Table 4**:** IL-8 Production by HUVEC, optical density (pg/ml), calculated concentration and concentration per 100ml

	L1			L2			L3			E			TNFα			McCoy			HUVEC Only		
	OD	Conc. (pg/ml)	Conc. /100	L2	Conc. (pg/ml)	Conc. /100	OD	Conc. (pg/ml)	Conc. /100	OD	Conc. (pg/ml)	Conc. /100	OD	Conc. (pg/ml)	Conc. /100	OD	Conc. (pg/ml)	Conc. /100	OD	Conc. (pg/ml)	Conc. /100
Exp 1	2.044	1437.5	0.299	1.799	1233.3	0.257	2.646	1939.2	0.404	2.19	1559.2	0.325	2.072	1460.83	0.304	1.933	1345	0.28	1.913	1328.3	0.27674
	1.987	1390	0.29	1.648	1107.5	0.231	2.014	1412.5	0.294	2.223	1586.7	0.331	1.991	1393.33	0.29	1.935	1346.7	0.281	1.626	1089.2	0.22691
	1.693	1145	0.239	1.834	1262.5	0.263	2.662	1952.5	0.407	2.024	1420.8	0.296	2.646	1939.17	0.404	2.003	1403.3	0.292	1.999	1400	0.29167
Exp 2	1.863	1337.6	0.248	2.195	1603.2	0.297	3.005	2251.2	0.417	2.19	1599.2	0.296	2.089	1518.4	0.281	1.632	1152.8	0.213	1.752	1248.8	0.23126
	2.27	1663.2	0.308	1.904	1370.4	0.254	2.668	1981.6	0.367	2.019	1462.4	0.271	2.402	1768.8	0.328	2.029	1470.4	0.272	1.907	1372.8	0.25422
	1.748	1245.6	0.231	1.991	1440	0.267	2.764	2058.4	0.381	2.094	1522.4	0.282	1.856	1332	0.247	1.492	1040.8	0.193	1.578	1109.6	0.20548
Exp 3	2.101	1383.8	0.216	1.814	1172.8	0.183	3.244	2224.3	0.348	1.94	1265.4	0.198	3.377	2322.06	0.363	2.078	1366.9	0.214	1.577	998.53	0.15602
	1.869	1213.2	0.19	1.692	1083.1	0.169	2.585	1739.7	0.272	2.154	1422.8	0.222	3.377	2322.06	0.363	1.921	1251.5	0.196	2.038	1337.5	0.20898
	2.991	2038.2	0.318	2.056	1350.7	0.211	3.378	2322.8	0.363	2.051	1347.1	0.21	3.377	2322.06	0.363	1.794	1158.1	0.181	1.804	1165.4	0.1821
Mean			0.26			0.237			0.361			0.27			0.327			0.236			0.22593
STDEV			0.043			0.039			0.047			0.046			0.047			0.042			0.04116
N			9			9			9			9			9			9			9
SEM			0.014			0.013			0.015			0.014			0.014			0.016			0.01372

Table 5: MCP-1 Production by HUVEC, optical density (pg/ml), calculated concentration and concentration per 100ml

	L1			L2			L3			E			TNFα			McCoy			HUVEC Only		
	OD	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**	**OD**	**Conc. (pg/ml)**	**Conc. /100**
Exp 1	0.308	152.22	0.0317	0.274	133.33	0.0278	0.475	245	0.051	0.341	170.56	0.0355	0.344	172.22	0.036	0.282	137.78	0.029	0.16	69.444	0.0145
	0.23	108.89	0.0227	0.273	132.78	0.0277	0.334	166.67	0.0347	0.425	217.22	0.0453	0.624	327.78	0.068	0.202	93.333	0.019	0.35	172.78	0.036
	0.269	130.56	0.0272	0.299	147.22	0.0307	0.33	164.44	0.0343	0.318	157.78	0.0329	0.527	273.89	0.057	0.479	247.22	0.052	0.4	202.78	0.0422
Exp 2	0.473	334.79	0.062	0.327	222.48	0.0412	0.173	104.02	0.0193	0.3	201.72	0.0374	0.47	332.48	0.062	0.181	110.18	0.02	0.11	54.023	0.01
	0.352	241.72	0.0448	0.279	185.56	0.0344	0.284	189.41	0.0351	0.287	191.72	0.0355	0.673	488.64	0.09	0.218	138.64	0.026	0.22	141.72	0.0262
	0.145	82.485	0.0153	0.226	144.79	0.0268	0.568	407.87	0.0755	0.322	218.64	0.0405	0.552	395.56	0.073	0.244	158.64	0.029	0.26	169.41	0.0314
Exp 3	0.217	182.56	0.0285	0.227	193.67	0.0303	0.417	404.78	0.0632	0.267	238.11	0.0372	0.46	452.56	0.071	0.258	228.11	0.036	0.1	49.222	0.0077
	0.327	304.78	0.0476	0.275	247	0.0386	0.377	360.33	0.0563	0.233	200.33	0.0313	0.594	601.44	0.094	0.226	192.56	0.03	0.16	115.89	0.0181
	0.36	341.44	0.0534	0.295	269.22	0.0421	0.499	495.89	0.0775	0.295	269.22	0.0421	0.563	567	0.089	0.22	185.89	0.029	0.18	142.56	0.0223
Mean			0.037			0.0333			0.0497			0.0375			0.071			0.03			0.0232
STDEV			0.0147			0.0057			0.0191			0.0042			0.017			0.009			0.0112
N			9			9			9			9			9			9			9
SEM			0.0049			0.0019			0.0064			0.0014			0.003			0.004			0.0058





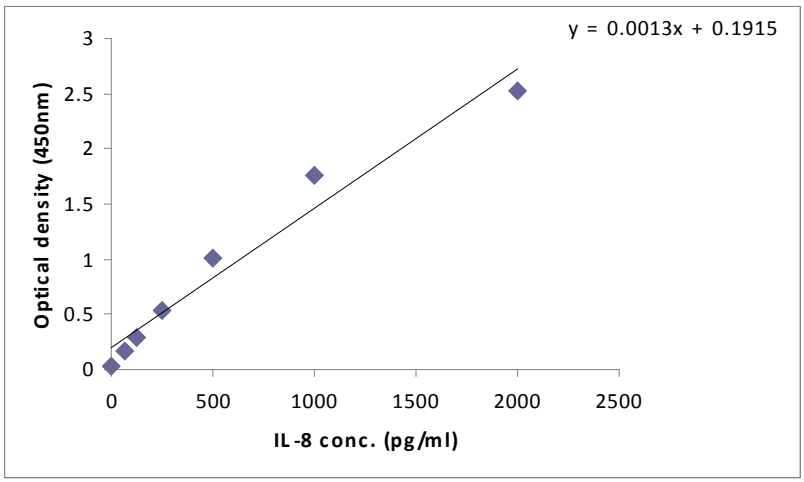
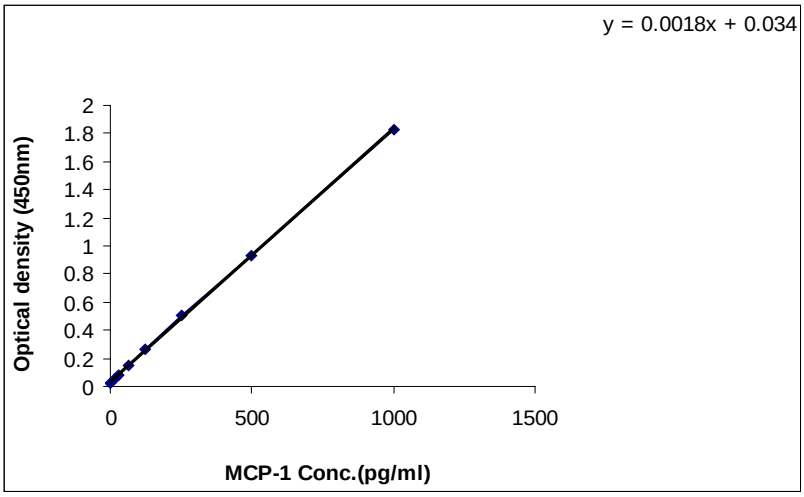
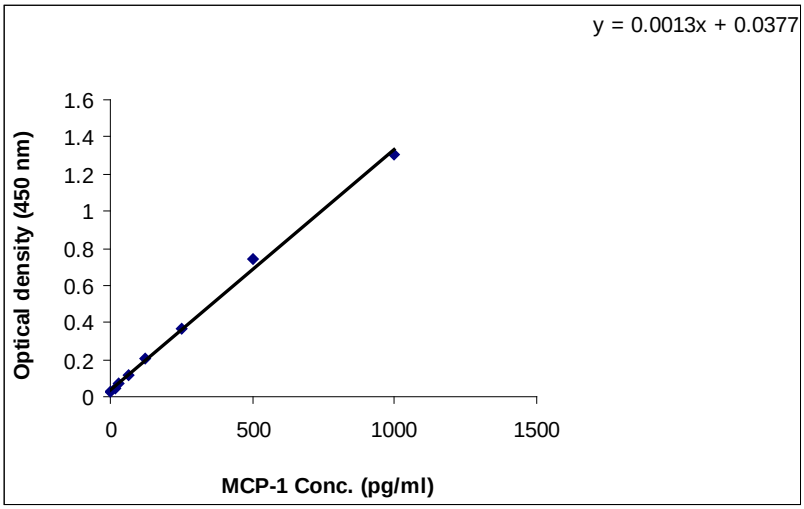


Figure 19: (A, B, C) Standard Curves for IL-8 triplicate experiments.





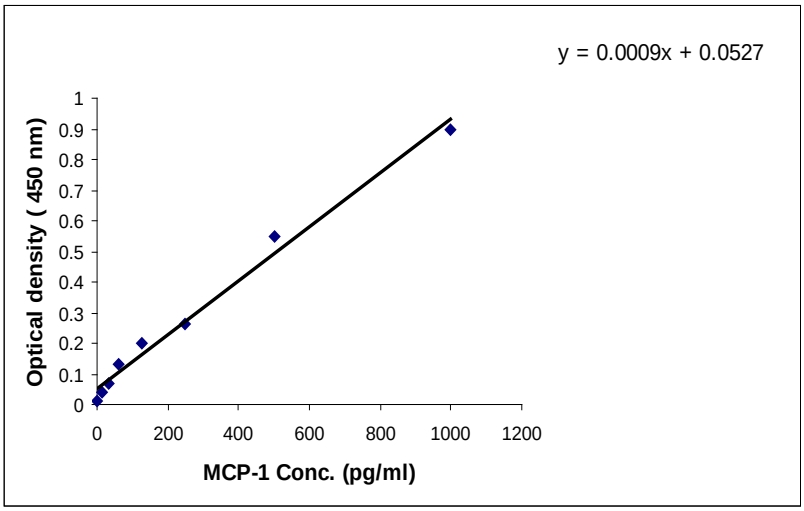


Figure 20: (A, B, C) Standard Curves for MCP-1 triplicate experiments.

Table 6: Optical density of ICAM-1 produced by HUVEC infected with *C. trachomatis* serovars and controls, measured at 450nm.

	L1	**L2**	**L3**	**E**	**TNFα**	**McCoy**	**HUVEC only**
Exp 1	1.125	0.79	0.877	0.971	1.189	0.729	1.084
	0.79	0.711	1.253	1.126	1.202	0.963	0.744
	0.853	0.799	0.864	0.649	1.153	0.946	0.877
Exp 2	0.981	1.017	1.562	0.944	1.116	0.655	0.755
	1.096	0.999	1.088	0.9	1.272	0.831	0.812
	1.113	0.933	1.291	0.877	1.124	0.816	0.956
Exp 3	0.839	0.756	0.971	0.854	1.14	0.798	0.793
	1.028	0.79	0.923	0.931	1.277	0.812	0.917
	0.857	0.844	0.855	0.84	1.243	0.857	0.791
Mean	0.964667	0.848778	1.076	0.899111	1.190667	0.823	0.858777778
STDEV	0.124543	0.102718	0.231352	0.119375	0.058636	0.090556	0.104855221
N	9	9	9	9	9	9	9
SEM	0.077117	0.034239	0.077117	0.039792	1.067406	0.030185	0.034962

Table 7: Raw data absorbance readings measured at 450nm for the LDH Cytotoxicity assay of HUVEC infected with different *C. trachomatis* LI, L2, L3 and E, also included are the controls.

	L1	L2	L3	E	TNFα	McCoy	HUVEC only	Cell max. LDH release	Volume Correction control	Spontaneous. release control	Culture medium control
Exp 1	0.293	0.275	0.462	0.408	0.556	0.275	0.422	0.767	0.145	0.161	0.164
	0.362	0.29	0.484	0.381	0.381	0.352	0.36	0.772	0.145	0.171	0.147
	0.41	0.465	0.473	0.476	0.422	0.345	0.313	0.65	0.132	0.228	0.132
Exp 2	0.429	0.419	0.555	0.458	0.649	0.481	0.486	0.844	0.159	0.142	0.105
	0.41	0.432	0.65	0.377	0.427	0.387	0.456	0.742	0.122	0.104	0.102
	0.48	0.446	0.514	0.406	0.723	0.408	0.277	0.856	0.144	0.132	0.11
Exp 3	0.455	0.607	0.648	0.378	0.63	0.456	0.445	1.086	0.12	0.107	0.112
	0.419	0.521	0.587	0.58	0.597	0.455	0.615	0.999	0.119	0.107	0.116
	0.388	0.622	0.569	0.448	0.75	0.568	0.555	0.742	0.115	0.121	0.107

Table 8: Values after subtraction of Culture Medium control, background control and average absorbance values from experimental wells from the LDH Cytotoxicity assay.

	L1	L2	L3	E	TNFα	McCoy	HUVEC Only
Exp 1	0.144	0.126	0.313	0.259	0.407	0.273	0.126
	0.213	0.141	0.335	0.232	0.232	0.211	0.203
	0.261	0.316	0.324	0.327	0.234	0.164	0.196
Exp 2	0.323	0.313	0.449	0.352	0.543	0.375	0.38
	0.304	0.326	0.544	0.271	0.321	0.281	0.349
	0.374	0.34	0.408	0.3	0.617	0.302	0.171
Exp 3	0.343	0.495	0.536	0.266	0.518	0.344	0.343
	0.307	0.409	0.475	0.468	0.485	0.343	0.503
	0.276	0.51	0.457	0.336	0.638	0.456	0.443

Table 9**:** Calculated % Cytotoxicity of different chlamydia serovars

	L1	L2	L3	E	TNFα	McCoy	HUVEC Only
Exp 1	19.2922	16.02541	49.9637	40.16334	67.02359	42.70417	16.02540835
	31.81488	18.74773	53.95644	35.26316	35.26316	31.45191	30
	40.52632	50.50817	51.96007	52.50454	35.62613	22.92196	28.72958258
Exp 2	44.06442	42.53067	63.38957	48.51227	77.80675	52.03988	52.80674847
	41.15031	44.52454	77.96012	36.08896	43.75767	37.6227	48.05214724
	51.8865	46.67178	57.10123	40.53681	89.15644	40.84356	20.75153374
Exp 3	40.89806	59.34466	64.32039	31.5534	62.13592	41.01942	40.89805825
	36.52913	48.90777	56.91748	56.06796	58.13107	40.89806	60.31553398
	32.76699	61.16505	54.73301	40.04854	76.69903	54.61165	53.03398058
Mean	37.65876	43.15842	58.92245	42.30433	60.6222	40.45703	38.95699924
STDEV	8.63491	14.98144	8.095329	7.807463	18.16513	9.048203	14.8128252
N	9	9	9	9	9	9	9
SEM	2.878303	4.993815	2.698443	2.602488	6.055044	3.016068	4.937608399

Table10: Number of cells migrated during the transendothelial migration assay, cell count done with the haemacytometer and trypan blue dye.

	Neutrophils			Monocytes		
	Exp 1	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp 3**
L1	4	5	6	3	3	4
L2	5	5	3	4	3	5
L3	6	7	4	5	4	4
E	3	4	4	2	1	4
TNFα	11	14	8	7	6	5
McCoy	4	5	2	1	2	2
HUVEC only	3	4	3	1	1	2

Table11: Calculated number of migrated cells from the cell count

	Neutrophils				Monocytes			
	Exp 1	**Exp 2**	**Exp 3**	**AVG**	**Exp 1**	**Exp 2**	**Exp 3**	**AVG**
L1	12000	15000	18000	15000	9000	9000	12000	10000
L2	15000	15000	9000	13000	12000	9000	15000	12000
L3	18000	21000	12000	17000	18000	12000	12000	14000
E	9000	12000	12000	11000	6000	3000	12000	7000
TNFα	33000	39000	24000	32000	21000	18000	15000	18000
McCoy	12000	15000	6000	11000	3000	6000	6000	5000
HUVEC only	9000	12000	9000	10000	3000	3000	6000	4000

APPENDIX E: Statistical Analysis

Interleukin-8 Statistical analysis

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 4.204 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
L1 vs L2	0.02296	1.501	ns P>0.05
L1 vs L3	-0.1016	6.641	*** P<0.001
L1 vs E	-0.01028	0.6723	ns P>0.05
L1 vs McCoy	0.02406	1.573	ns P>0.05
L1 vs HUVEC Only	0.03387	2.215	ns P>0.05
L2 vs L3	-0.1245	8.142	*** P<0.001
L2 vs E	-0.03325	2.174	ns P>0.05
L2 vs McCoy	0.001094	0.07152	ns P>0.05
L2 vs HUVEC Only	0.01091	0.7132	ns P>0.05
L3 vs E	0.09129	5.968	** P<0.01
L3 vs McCoy	0.1256	8.214	*** P<0.001
L3 vs HUVEC Only	0.1354	8.855	*** P<0.001
E vs McCoy	0.03434	2.245	ns P>0.05
E vs HUVEC Only	0.04416	2.887	ns P>0.05
McCoy vs HUVEC Only	0.009815	0.6417	ns P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
L1 - L2	0.02296	-0.04134	0.08727
L1 - L3	-0.1016	-0.1659	-0.03726
L1 - E	-0.01028	-0.07459	0.05402
L1 - McCoy	0.02406	-0.04025	0.08837
L1 - HUVEC Only	0.03387	-0.03043	0.09818
L2 - L3	-0.1245	-0.1888	-0.06023
L2 - E	-0.03325	-0.09756	0.03106
L2 - McCoy	0.001094	-0.06321	0.06540
L2 - HUVEC Only	0.01091	-0.05340	0.07522
L3 - E	0.09129	0.02698	0.1556
L3 - McCoy	0.1256	0.06132	0.1899
L3 - HUVEC Only	0.1354	0.07114	0.1998
E - McCoy	0.03434	-0.02997	0.09865
E - HUVEC Only	0.04416	-0.02015	0.1085
McCoy - HUVEC Only	0.009815	-0.05449	0.07412

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 0.3654
The P value is 0.9962.
Bartlett's test suggests that the differences among the SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	0.1884	>0.10	Yes
L2	0.2127	>0.10	Yes
L3	0.1791	>0.10	Yes
E	0.1726	>0.10	Yes
McCoy	0.2456	>0.10	Yes
HUVEC Only	0.1181	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	0.1126	0.02253
Residuals (within columns)	48	0.1011	0.002106
Total	53	0.2137	

F = 10.700 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
L1	9	0.2598	0.04542	0.01514	0.2477
L2	9	0.2368	0.04186	0.01395	0.2538
L3	9	0.3614	0.05012	0.01671	0.3670
E	9	0.2701	0.04901	0.01634	0.2819
McCoy	9	0.2357	0.04470	0.01490	0.2136
HUVEC Only	9	0.2259	0.04366	0.01455	0.2269

Group	Minimum	Maximum	95% Confidence Interval From	To
L1	0.1896	0.3185	0.2249	0.2947
L2	0.1692	0.2969	0.2047	0.2690
L3	0.2718	0.4169	0.3228	0.3999
E	0.1977	0.3306	0.2324	0.3078
McCoy	0.1810	0.2924	0.2014	0.2701
HUVEC Only	0.1560	0.2917	0.1924	0.2595

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Monocyte Chemtactic Protein-1 Statistical analysis

One-way Analysis of Variance (ANOVA)

The P value is 0.0018, considered very significant.
Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 4.204 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
L1 vs L2	0.003747	0.8995	ns P>0.05
L1 vs L3	-0.01264	3.035	ns P>0.05
L1 vs E	-0.0004941	0.1186	ns P>0.05
L1 vs McCoy	0.007028	1.687	ns P>0.05
L1 vs HUVEC Only	0.01386	3.327	ns P>0.05
L2 vs L3	-0.01639	3.935	ns P>0.05
L2 vs E	-0.004241	1.018	ns P>0.05
L2 vs McCoy	0.003281	0.7876	ns P>0.05
L2 vs HUVEC Only	0.01011	2.428	ns P>0.05
L3 vs E	0.01215	2.917	ns P>0.05
L3 vs McCoy	0.01967	4.723	* P<0.05
L3 vs HUVEC Only	0.02650	6.363	*** P<0.001
E vs McCoy	0.007522	1.806	ns P>0.05
E vs HUVEC Only	0.01435	3.446	ns P>0.05
McCoy vs HUVEC Only	0.006831	1.640	ns P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
L1 - L2	0.003747	-0.01377	0.02126
L1 - L3	-0.01264	-0.03016	0.004869
L1 - E	-0.0004941	-0.01801	0.01702
L1 - McCoy	0.007028	-0.01049	0.02454
L1 - HUVEC Only	0.01386	-0.003654	0.03137
L2 - L3	-0.01639	-0.03390	0.001122
L2 - E	-0.004241	-0.02175	0.01327
L2 - McCoy	0.003281	-0.01423	0.02079
L2 - HUVEC Only	0.01011	-0.007401	0.02762
L3 - E	0.01215	-0.005363	0.02966
L3 - McCoy	0.01967	0.002158	0.03718
L3 - HUVEC Only	0.02650	0.008990	0.04402
E - McCoy	0.007522	-0.009991	0.02503
E - HUVEC Only	0.01435	-0.003160	0.03187
McCoy - HUVEC Only	0.006831	-0.01068	0.02434

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 21.305
The P value is 0.0007.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider

transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	0.1888	>0.10	Yes
L2	0.2227	>0.10	Yes
L3	0.2090	>0.10	Yes
E	0.1805	>0.10	Yes
McCoy	0.2736	0.0508	Yes
HUVEC Only	0.1096	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	0.003542	0.0007084
Residuals (within columns)	48	0.007495	0.0001562
Total	53	0.01104	

F = 4.537 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
L1	9	0.03701	0.01556	0.005188	0.03171
L2	9	0.03327	0.006010	0.002003	0.03067
L3	9	0.04966	0.02022	0.006740	0.05104
E	9	0.03751	0.004440	0.001480	0.03720
McCoy	9	0.02999	0.009484	0.003161	0.02905
HUVEC Only	9	0.02316	0.01183	0.003944	0.02227

Group	Minimum	Maximum	95% Confidence Interval From	To
L1	0.01527	0.06200	0.02505	0.04898
L2	0.02681	0.04207	0.02865	0.03789
L3	0.01926	0.07748	0.03412	0.06520
E	0.03130	0.04525	0.03410	0.04092
McCoy	0.01944	0.05150	0.02270	0.03728
HUVEC Only	0.007691	0.04225	0.01406	0.03225

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Intercellular Cell Adhesion Molecule-1 Statistical analysis

One-way Analysis of Variance (ANOVA)

The P value is 0.0057, considered very significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.204 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
L1 vs L2	0.1159	2.388 ns	P>0.05
L1 vs L3	-0.1113	2.294 ns	P>0.05
L1 vs E	0.06556	1.351 ns	P>0.05
L1 vs McCoy	0.1417	2.919 ns	P>0.05
L1 vs HUVEC only	0.1059	2.182 ns	P>0.05
L2 vs L3	-0.2272	4.682 *	P<0.05
L2 vs E	-0.05033	1.037 ns	P>0.05
L2 vs McCoy	0.02578	0.5312 ns	P>0.05
L2 vs HUVEC only	-0.01000	0.2061 ns	P>0.05
L3 vs E	0.1769	3.645 ns	P>0.05
L3 vs McCoy	0.2530	5.214 **	P<0.01
L3 vs HUVEC only	0.2172	4.476 *	P<0.05
E vs McCoy	0.07611	1.568 ns	P>0.05
E vs HUVEC only	0.04033	0.8312 ns	P>0.05
McCoy vs HUVEC only	-0.03578	0.7373 ns	P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
L1 - L2	0.1159	-0.08814	0.3199
L1 - L3	-0.1113	-0.3154	0.09269
L1 - E	0.06556	-0.1385	0.2696
L1 - McCoy	0.1417	-0.06236	0.3457
L1 - HUVEC only	0.1059	-0.09814	0.3099
L2 - L3	-0.2272	-0.4312	-0.02320
L2 - E	-0.05033	-0.2544	0.1537
L2 - McCoy	0.02578	-0.1782	0.2298
L2 - HUVEC only	-0.01000	-0.2140	0.1940
L3 - E	0.1769	-0.02714	0.3809

L3 - McCoy	0.2530	0.04898	0.4570
L3 - HUVEC only	0.2172	0.01320	0.4212
E - McCoy	0.07611	-0.1279	0.2801
E - HUVEC only	0.04033	-0.1637	0.2444
McCoy - HUVEC only	-0.03578	-0.2398	0.1682

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 10.522

The P value is 0.0617.

Bartlett's test suggests that the differences among the SDs is not quite significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method

Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	0.2369	>0.10	Yes
L2	0.2317	>0.10	Yes
L3	0.2212	>0.10	Yes
E	0.2092	>0.10	Yes
McCoy	0.1751	>0.10	Yes
HUVEC only	0.2185	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	0.4013	0.08025
Residuals (within columns)	48	1.017	0.02119
Total	53	1.419	

F = 3.787 = (MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
L1	9	0.9647	0.1321	0.04403	0.9810
L2	9	0.8488	0.1089	0.03632	0.7990
L3	9	1.076	0.2454	0.08180	0.9710
E	9	0.8991	0.1266	0.04221	0.9000
McCoy	9	0.8230	0.09605	0.03202	0.8160
HUVEC only	9	0.8588	0.1112	0.03707	0.8120

95% Confidence Interval

Group	Minimum	Maximum	From	To
L1	0.7900	1.125	0.8631	1.066
L2	0.7110	1.017	0.7650	0.9325
L3	0.8550	1.562	0.8874	1.265
E	0.6490	1.126	0.8018	0.9964
McCoy	0.6550	0.9630	0.7492	0.8968
HUVEC only	0.7440	1.084	0.7733	0.9443

Transendothelial migration assay statistical analysis

Neutrophils

One-way Analysis of Variance (ANOVA)

The P value is 0.1642, considered not significant.

Variation among column means is not significantly greater than expected by chance.

Post tests

Post tests were not calculated because the P value was greater than 0.05.

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical

SDs. This assumption is tested using the method of Bartlett.

Bartlett's test can only be performed when every column has at least five values.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian

distributions. This assumption is tested using the method

Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	Too few values to test.		
L2	Too few values to test.		
L3	Too few values to test.		
E	Too few values to test.		
McCoy	Too few values to test.		
HUVEC only	Too few values to test.		

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	1.105	2.210
Residuals (within columns)	12	1.380	1.150

Total 17 2.485

F = 1.922 = (MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
L1	3	15000	3000.0	1732.1	15000
L2	3	13000	3464.1	2000.0	15000
L3	3	17000	4582.6	2645.8	18000
E	3	11000	1732.1	1000.0	12000
McCoy	3	11000	4582.6	2645.8	12000
HUVEC only	3	10000	1732.1	1000.0	9000.0

			95% Confidence Interval	
Group	Min	Max	From	To
L1	12000	18000	7547.0	22453
L2	9000.0	15000	4394.0	21606
L3	12000	21000	5615.3	28385
E	9000.0	12000	6697.0	15303
McCoy	6000.0	15000	-384.67	22385
HUVEC only	9000.0	12000	5697.0	14303

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Monocytes

One-way Analysis of Variance (ANOVA)

The P value is 0.0069, considered very significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.751 then the P value is less

than 0.05.

Comparison	Mean Difference	q	P value
L1 vs L2	-2000.0	1.188	ns P>0.05
L1 vs L3	-4000.0	2.376	ns P>0.05
L1 vs E	3000.0	1.782	ns P>0.05
L1 vs McCoy	5000.0	2.970	ns P>0.05
L1 vs HUVEC only	6000.0	3.565	ns P>0.05
L2 vs L3	-2000.0	1.188	ns P>0.05
L2 vs E	5000.0	2.970	ns P>0.05
L2 vs McCoy	7000.0	4.159	ns P>0.05
L2 vs HUVEC only	8000.0	4.753	* P<0.05
L3 vs E	7000.0	4.159	ns P>0.05
L3 vs McCoy	9000.0	5.347	* P<0.05
L3 vs HUVEC only	10000	5.941	* P<0.05
E vs McCoy	2000.0	1.188	ns P>0.05
E vs HUVEC only	3000.0	1.782	ns P>0.05
McCoy vs HUVEC only	1000.0	0.5941	ns P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
L1 - L2	-2000.0	-9997.1	5997.1
L1 - L3	-4000.0	-11997	3997.1
L1 - E	3000.0	-4997.1	10997
L1 - McCoy	5000.0	-2997.1	12997
L1 - HUVEC only	6000.0	-1997.1	13997
L2 - L3	-2000.0	-9997.1	5997.1
L2 - E	5000.0	-2997.1	12997
L2 - McCoy	7000.0	-997.12	14997
L2 - HUVEC only	8000.0	2.875	15997
L3 - E	7000.0	-997.12	14997
L3 - McCoy	9000.0	1002.9	16997
L3 - HUVEC only	10000	2002.9	17997
E - McCoy	2000.0	-5997.1	9997.1
E - HUVEC only	3000.0	-4997.1	10997
McCoy - HUVEC only	1000.0	-6997.1	8997.1

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett's test can only be performed when every column has at least five values.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	Too few values to test.		
L2	Too few values to test.		
L3	Too few values to test.		
E	Too few values to test.		
McCoy	Too few values to test.		
HUVEC only	Too few values to test.		

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	2.380	4.760
Residuals (within columns)	12	1.020	8500000
Total	17		3.400

F = 5.600 = (MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
L1	3	10000	1732.1	1000.0	9000.0
L2	3	12000	3000.0	1732.1	12000
L3	3	14000	3464.1	2000.0	12000
E	3	7000.0	4582.6	2645.8	6000.0
McCoy	3	5000.0	1732.1	1000.0	6000.0
HUVEC only	3	4000.0	1732.1	1000.0	3000.0

			95% Confidence Interval	
Group	Minimum	Maximum	From	To
L1	9000.0	12000	5697.0	14303
L2	9000.0	15000	4547.0	19453
L3	12000	18000	5394.0	22606
E	3000.0	12000	-4384.7	18385
McCoy	3000.0	6000.0	697.00	9303.0
HUVEC only	3000.0	6000.0	-303.00	8303.0

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LDH cytotoxicity assay statistical analysis

One-way Analysis of Variance (ANOVA)

The P value is 0.0004, considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.329 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
L1 vs L2	-5.500	1.266 ns	P>0.05
L1 vs L3	-21.264	4.895 *	P<0.05
L1 vs E	-4.646	1.069 ns	P>0.05
L1 vs TNF-alpha	-22.963	5.286 **	P<0.01
L1 vs McCoy	-2.798	0.6442 ns	P>0.05
L1 vs HUVEC Only	-1.298	0.2989 ns	P>0.05
L2 vs L3	-15.764	3.629 ns	P>0.05
L2 vs E	0.8541	0.1966 ns	P>0.05
L2 vs TNF-alpha	-17.464	4.020 ns	P>0.05
L2 vs McCoy	2.701	0.6219 ns	P>0.05
L2 vs HUVEC Only	4.201	0.9672 ns	P>0.05
L3 vs E	16.618	3.825 ns	P>0.05
L3 vs TNF-alpha	-1.700	0.3913 ns	P>0.05
L3 vs McCoy	18.465	4.251 ns	P>0.05
L3 vs HUVEC Only	19.965	4.596 *	P<0.05
E vs TNF-alpha	-18.318	4.217 ns	P>0.05
E vs McCoy	1.847	0.4252 ns	P>0.05
E vs HUVEC Only	3.347	0.7706 ns	P>0.05
TNF-alpha vs McCoy	20.165	4.642 *	P<0.05
TNF-alpha vs HUVEC Only	21.665	4.987 *	P<0.05
McCoy vs HUVEC Only	1.500	0.3453 ns	P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
L1 - L2	-5.500	-24.305	13.306
L1 - L3	-21.264	-40.069	-2.458
L1 - E	-4.646	-23.451	14.160
L1 - TNF-alpha	-22.963	-41.769	-4.158
L1 - McCoy	-2.798	-21.604	16.007
L1 - HUVEC Only	-1.298	-20.104	17.507
L2 - L3	-15.764	-34.569	3.041
L2 - E	0.8541	-17.951	19.659
L2 - TNF-alpha	-17.464	-36.269	1.342
L2 - McCoy	2.701	-16.104	21.507
L2 - HUVEC Only	4.201	-14.604	23.007
L3 - E	16.618	-2.187	35.423
L3 - TNF-alpha	-1.700	-20.505	17.106
L3 - McCoy	18.465	-0.3399	37.271
L3 - HUVEC Only	19.965	1.160	38.771
E - TNF-alpha	-18.318	-37.123	0.4875
E - McCoy	1.847	-16.958	20.653
E - HUVEC Only	3.347	-15.458	22.153
TNF-alpha - McCoy	20.165	1.360	38.971
TNF-alpha - HUVEC Only	21.665	2.860	40.471
McCoy - HUVEC Only	1.500	-17.305	20.305

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 11.502

The P value is 0.0740.

Bartlett's test suggests that the differences among the SDs is not quite significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
L1	0.1784	>0.10	Yes
L2	0.2620	>0.10	Yes
L3	0.2506	>0.10	Yes
E	0.2512	>0.10	Yes
TNF-alpha	0.1426	>0.10	Yes
McCoy	0.1852	>0.10	Yes
HUVEC Only	0.1631	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	6	4971.9	828.65
Residuals (within columns)	56	9510.8	169.84
Total	62	14483	

F = 4.879 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median		
L1	9	37.659	9.159	3.053	40.526		
L2	9	43.158	15.890	5.297	46.672		
L3	9	58.922	8.586	2.862	56.917		
E	9	42.304	8.281	2.760	40.163		
TNF-alpha	9	60.622	19.267	6.422	62.136		
McCoy	9	40.457	9.597	3.199	40.898		
HUVEC Only	9	38.957	15.711	5.237	40.898		

Group	Minimum	Maximum	95% Confidence Interval From	To
L1	19.292	51.887	30.619	44.699
L2	16.025	61.165	30.944	55.373
L3	49.964	77.960	52.322	65.523
E	31.553	56.068	35.939	48.670
TNF-alpha	35.263	89.156	45.812	75.432
McCoy	22.922	54.612	33.080	47.834
HUVEC Only	16.025	60.316	26.880	51.034

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