Investigating the Effects of *Yersinia pestis* V antigen as an Immunomodulator of Innate Immune Responses in Sepsis

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To Dad, who gave me everything

this one's for you

Summary

Sepsis is not only the leading cause of death in non-coronary intensive care units (ICUs) but also one of the most common causes of morbidity and mortality for all hospitalised patients. Globally, 20 to 30 million patients are estimated to be afflicted every year with an astonishing hospital mortality rates between 30 and 60%. There is no current therapy for sepsis other than anti-infectives and supportive care. These approaches only give the body time to recover, but do not treat the cause of the problem.

In this study we seek to discover the effects of the virulence factor from a bacterium, *Yersinia pestis* V antigen on LPS-induced responses. *Y. pestis*, the causative agent of the three main plague pandemics has been responsible for over 200 million deaths. The bacterium has been so successful because it encodes several virulence factors, one of which is V antigen.

Our results demonstrate a modulatory role of *Y. pestis* V antigen on bacterial infections. Using monocytic and macrophage cells we have shown that V antigen can reduce the expression of pattern recognition receptors (PRRs) of the innate immune system, causing the modulation of the cellular response directed towards LPS bacterial pathogen-associated molecular patterns (PAMPs). We also tested different fractions of *Y. pestis* in order to identify the functional domain of responsible for this immunomodulation. Our results demonstrated that this functional domain in found within the amino acids 135-275 of V antigen.

Of a greater magnitude, our *in vivo* data show an impressive 80% reduction in mortality in a mouse model of sepsis when mice are treated with V antigen in comparison to those that were not. This protein should unquestionably be further investigated as a possible therapeutic intervention for sepsis.

Abbreviations

Ab	Antibody
AD	*Anno Domini*
AIDS	Acquired immunodeficiency syndrome
AIM2	Absent in melanoma 2
APACHE II	Acute Physiology and Chronic Heath Evaluation II
APC	Activated protein C
APS	Ammonium Persulphate
ARDS	Acute respiratory distress syndrome
ASC	Apoptosis-associated speck-like protein containing CARD
ASPA	Animals (Scientific Procedures) Act 1986
AT III	Antithrombin III
BC	Before Christ
CARD	Caspase activation and recruitment domain
CBA	Cytometric bead array
CDC	Centers for Disease
CO_2	Carbon dioxide
CORTICUS	Corticosteroid Therapy of Septic Shock
CpG	Cytosine-phosphate-guanosine
CXCR4	CX Chemokine receptor 4
Cy3	Cyanine 5
Cy5	Cyanine 3
Da	Dalton
DAMP	Damage-associated molecular patterns
DCs	Dendritic cells
DD	Death domain
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's Modified Eagle Medium
DMP	Dexamethasone
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded-RNA
dToll	*Drosophila* Toll
E. coli	*Escherichia coli*

ECA	Enterobacterial common antigen
ECACC	European Collection of Animal Cell Cultures
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
EUPHAS	Early Use of Polymyxin B Hemoperfusion in Abdominal Sepsis
EUPHRATES	Evaluating the Use of Polymyxin B Hemoperfusion in a Randomized controlled trial of Adults Treated for Endotoxemia and Septic shock
Fab	Fragment antigen-binding region
FACS	Fluorescent Activated Cell Sorting
Fc	Fragment crystallisable region
FCS	Foetal calf serum
FDA	Food and Drug Administration
FIIND	Function-to-find domain
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
GST	Glutathione S-transferase
HIV	Human immunodeficiency virus
HRP	Horse-radish peroxidase
Hsps	Heat shock proteins
hTLR	Human TLR
i.p.	Intraperitoneal
ICD-9-CM	International Classification of Diseases, Ninth Revision, Clinical Modification
ICNARC	Intensive Care National Audit & Research Centre
ICU	Intense care unit
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFI16	IFN-γ-inducible protein 16
IFN	Interferon
IKK	IκB-kinase
IL	Interleukin

IL-1R	Interlukin-1 receptor
IL-1ra	IL-1 receptor antagonist
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK	IL-1R-associated kinase
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
L,D-Hep	L-*glycero*-D-*manno*heptose
LAL	*Limulus* Amebocyte Lysate
LBP	LPS-binding protein
Lcr	Low-calcium response
LcrV	V antigen
L-NAME	N-omega-nitro-L-arginine methyl ester
L-NMMA	L-N-monomethyl argenine
LPS	Lipopolysaccharide
LSM	Laser scanning microscope
LT	Lethal toxin
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MAL	MyD88-adaptor like
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation factor 2
MDP	Muramyl dipeptide
MPS	Methylprednisolone
mTLR	Mouse TLR
MW	Molecular weight
MyD88	Myeloid differentiation primary gene-88
NACHT	Nucleotide-binding and oligomerisation
NBD-LRR	Nucleotide-binding domain leucine-rich repeat-containing
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NLRC	NOD-, LRR- and CARD-containing
NLRP	NOD-, LRR- and pyrin domain-containing
NLRs	NOD-like receptors

NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
NOS	Nitric oxide synthase
NSADs	Non-steroidal anti-inflammatory drugs
OG	Oregon green
PAF	Platelet-activating factor
PAI	Plasminogen activator inhibitor
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PD	Phosphodiester
PE	Phycoerythrin
PFA	Paraformaldehyde
PLA	Plasminogen activator
PLA_2	Phospholipase A_2
PMX	Polymyxin B
PMX-DHP	PMX direct hemoperfusion
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PS	Phorothionate
PYD	Pyrin domain
PYTHIN	Pyrin and HIN domain-containing protein
RAW 264.7	Mouse leukaemic monocyte macrophage
$rBPI_{21}$	Recombinant bactericidal/permeability-increasing protein
rhAPC	Recombinant human activated protein C
rhTM	Recombinant human soluble thrombomodulin
RIP-1	Receptor-interacting protein 1
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
SCC	Side scatter
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRS	Systemic inflammatory response syndrome
SOPA	Sepsis Occurrence in Acutely Ill Patients
ssDNA	Single-stranded-DNA
T3S	Type III secretion system

TAB	TAK1 binding proteins
TAFI	Thrombin-activatable fibrinolysis inhibitor
TANK	TRAF family member-associated NF-κB activator
TBK	TRAF family member-associated NF-κB activator binding kinase
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Tissue factor
TFPI	Anti factor pathway inhibitor
THP-1	Human monocytic leukemia
TIR	Toll/IL-1 receptor
TIRAP	TIR-domain containing adaptor protein
TLF	Talactoferrin alfa
TLRs	Toll-like receptors
TM	Thrombomodulin
TNF	Tumour necrosis factor
TNFR	TNF receptor
t-PA	Plasminogen activation factor
TRADD	TNF receptor-associated death domain
TRAF	Tumor necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor-inducing interferon-β
V antigen	Virulence antigen
WHO	World Health Organisation
Y. enterocolitica	*Yersinia enterocolitica*
Y. pestis	*Yersinia pestis*
Y. pseudotuberculosis	*Yersinia pseudotuberculosis*
Yops	*Yersinia* outer proteins

Contents

Chapter 1

Introduction

1.1. Sepsis

Sepsis is one of the oldest and most puzzling conditions in medical history. Sepsis (σηψις), which is the original Greek word for the "decomposition of animal or vegetable organic matter in the presence of bacteria" was described by Hippocrates as the process by which flesh rots, generating foul odours and wound putrefaction (Majno, 1991). The term sepsis is found in the Corpus Hippocraticum, but its earliest appearance is in Homer's poems, where sepsis is derived of the verb form sepo (σηπω), meaning "I rot". Aristoteles, Plutarch and Galen use sepsis in the same sense as Hippocrates. For 2,700 years the word "sepsis" has essentially maintained its core meaning (Geroulanos and Douka, 2006).

Understanding of this condition took a great leap forward when the schools of Hippocrates (460-370 BC) and Galenos (Galen of Pergamum; AD 129-199) concluded that a poison was the dominant cause.

Figure 1.1: Thirteen century fresco of Hippocrates (right) and Galenos (left). These two masters dominated European medical education and practice until the seventeenth century. The text on the paper in front of Galenos reads "Mundi presentis seres manet ex elementis", meaning "The present world's connection persists on the basis of the elements". The text on the paper in front of Hippocrates reads "Ex his formantur que sunt quecu(m)q(ue) chreantur", meaning "Of these (all) is formed which exists and which will be created" (Beutler and Rietschel, 2003).

Experiments preformed by Albrecht von Haller (1708-1777) and François Magendie (1783-1855) supported the hypothesis that poison was present in putrid matter. They showed that intravenous application of decomposed fish or meat to experimental animals caused symptoms of illness. It was Louis Pasteur (1822-1895) that conclusively linked the decay of organic matter to the presence of bacteria and microorganisms. He recognised that microbes were not only necessary, but sufficient, to cause an infectious disease (Beutler and Rietschel, 2003). In spite of this, the presence of bacteria did not entirely explain the pathogenesis of sepsis. Many patients with sepsis died despite the successful eradication of pathogens with the use of modern antibiotics. This lead researchers to suggest that it was the host, not the bacteria, that drove the pathogenesis of sepsis (Cerra, 1985).

1.1.1. Definitions

Systemic inflammatory response to infection has long been surrounded by confusion. Several terms have been used interchangeably: septicaemia, sepsis, sepsis syndrome and septic shock. In clinical practice, sepsis is a particularly unsettled term used in describing the body's systemic response to infection. In 1991, The American College of Chest Physicians and the Society of Critical Care Medicine held a Consensus Conference with the aim of publishing the definitions of sepsis syndromes to clarify the terminology used to describe the body's systemic response to infection (Bone *et al.*, 1992). The panel defined sepsis as a systemic inflammatory response to infection noting that sepsis could occur in response to multiple infectious causes. This lead to the proposal of the phrase systemic inflammatory response syndrome (SIRS) which describes a systemic inflammatory response, independent of its cause. The term septicaemia was deemed both unnecessary and unhelpful which lead to its elimination from current usage. The panel coined the terms "severe sepsis" to describe when sepsis is associated with organ dysfunction, and "septic shock" for sepsis complicated by hypotension that is refractory to fluid resuscitation (Bone *et al.*, 1992).

Bacteraemia	The presence of viable bacteria in the blood
SIRS	The systemic inflammatory response to a variety of severe clinical insults which is manifested by two or more of the following conditions: 1. Temperature >38ºC or <36ºC 2. Heart rate >90 beats per minute 3. Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mm Hg 4. White blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms.
Sepsis	The systemic inflammatory response (SIRS) as a result of infection.
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension.
Septic shock	Sepsis-induced with hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status.

Table 1.1: Definitions of bacteraemia, SIRS, sepsis, severe sepsis and septic shock (Bone *et al.*, 1992).

In 2001, a second panel, the International Sepsis Definitions Conference, revisited the 1992 sepsis guidelines. Based on this conference a consensus document was developed (Levy *et al.*, 2003). This document concluded that there was not enough evidence to support a change to the previous definitions aside from expanding the list of signs and symptoms of sepsis to reflect the clinical bedside experience.

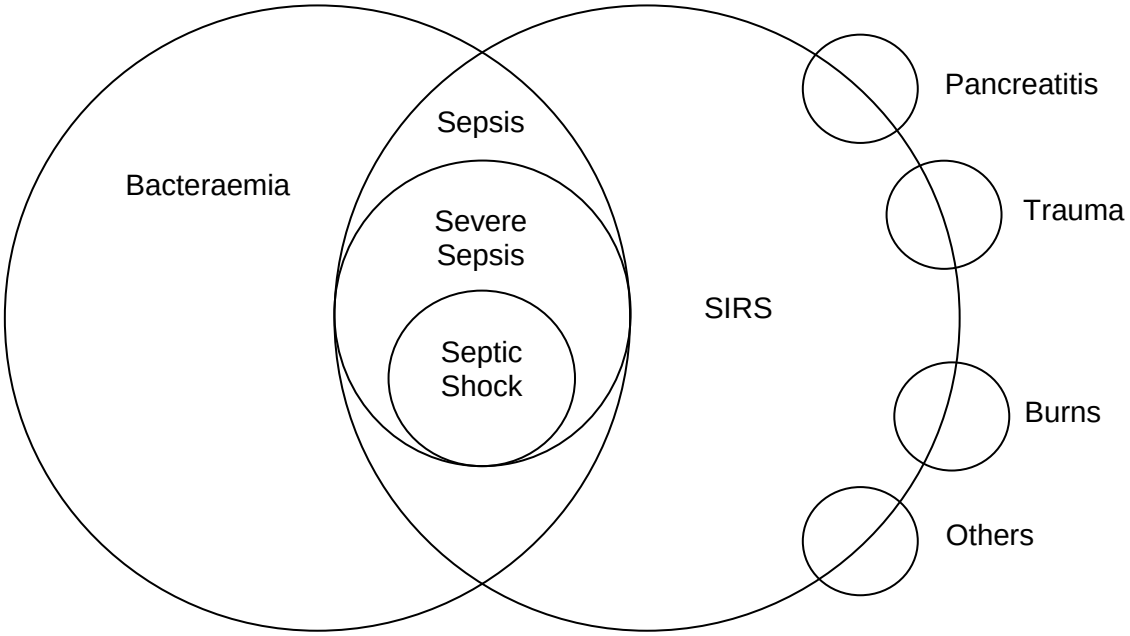


Figure 1.2: The relationship between infection, SIRS, sepsis, severe sepsis and septic shock. Adapted from Bone *et al.* (1992).

1.1.2. Incidence and causes

The epidemiology of sepsis, severe sepsis and septic shock is not well known due to the absence of population based prospective cohort studies. Most of the studies on the epidemiology of sepsis are based on hospital discharge diagnoses. In 1990, the Centers for Disease and Control (CDC), based on data from the National Hospital Discharge Survey, estimated that in the United States there were 450,000 cases of sepsis per year (Centers for Disease, 1990). In 1995, based on the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes, Angus *et al.*, in a large observational cohort study (n=6,621,559) identified 751,000 cases of severe sepsis annually, representing an estimate of 3 cases per 1,000 population and 2.26 cases per 100 hospital discharges (Angus *et al.*, 2001). Using the National Hospital Discharge Survey database, Martin and co-workers identified 10,319,418 cases of sepsis from an estimated 750 million hospitalizations in the United

States over a 22-year period, with an increase of frequency from 82.7 cases per 100,000 population in 1979 to 240.4 cases per 100,000 population in 2000, meaning there was an annualized increase in the incidence of sepsis of 8.7% (Martin *et al.*, 2003).

In European intensive care units, a Sepsis Occurrence in Acutely Ill Patients (SOPA) study collected data on all patients admitted to the intense care units (ICUs) during a two week period. A total of 3,147 adult patients were enrolled and 1,177 (37.4%) were diagnosed with sepsis (Vincent *et al.*, 2006).

Padkin and colleagues investigated the occurrence of severe sepsis in the first 24 hours of admission in 91 general ICUs in England, Wales and Northern Ireland between 1995 and 2000. They found that from 56,673 adult admissions to ICUs 15,362 (27.1%) had severe sepsis in the first 24 hours (Padkin *et al.*, 2003). Using the Intensive Care National Audit & Research Centre (ICNARC) Case Mix Programme Database, Harrison et al. (2006) evaluated the admissions with severe sepsis occurring at any time within 24 hours of admissions to ICUs in England, Wales and Northern Ireland, between 1995 and 2005. From 342,860 admissions, 92,672 admissions (27%) were identified as having severe sepsis in the first 24 hours following admission. Harrison and co-workers also identified that the percentage of admission with severe sepsis during the first 24 hours increased from 23.5% in 1996 to 28.7% in 2004. This represents an estimated 31,000 annual admissions to all 240 adult general critical care units in England, Wales and Northern Ireland (Harrison *et al.*, 2006).

Severe sepsis can arise as a result of both public-acquired and health care-linked infections. Respiratory tract infections are the most common site of infection both in severe sepsis and in septic shock, followed by genitourinary and intraabdominal infections (Angus *et al.*, 2001, Esper *et al.*, 2006, Mayr *et al.*, 2010, Vincent *et al.*, 2009). Blood cultures from patients with severe sepsis and septic shock are positive in

only one third of cases. This might occur due to the increase in empiric antibiotic treatment. In positive blood cultures, the most common Gram-positive microorganisms present are *Staphylococcus aureus* and *Streptococcus pneumonia*, whereas *Escherichia coli, Klebsiella* spp. and *Pseudomonas aeruginosa* are the more prevalent Gram-negative isolates (Angus *et al.*, 2001). Martin *et al.* (2003) in an epidemiology study of sepsis from 1979 through 2000, showed that Gram-positive bacteria became the predominant organisms causing sepsis in comparison to Gram-negative infections. In spite of this, in a more recent study conducted by Vincent and colleagues (2009) involving 14,414 patients in 1265 ICUs from 75 countries, 62% of the positive isolates were Gram-negative organisms, 47% were Gram-positive and 19% were fungi (Vincent *et al.*, 2009).

Age, race and sex also influence the incidence of severe sepsis. The incidence of severe sepsis and septic shock is higher in infants and elderly persons than in other age groups, with a sharp increase in incidence in elderly people (Angus *et al.*, 2001, Dombrovskiy *et al.*, 2007, Harrison *et al.*, 2006, Martin *et al.*, 2003, Padkin *et al.*, 2003, Vincent *et al.*, 2006, Wang *et al.*, 2007). Epidemiologic studies have shown a higher incidence of severe sepsis and septic shock in black people (Martin *et al.*, 2003, Mayr *et al.*, 2010). Several reports have also shown that men are more likely to develop sepsis than women (Angus *et al.*, 2001, Bateman *et al.*, 2010, Martin *et al.*, 2003, Padkin *et al.*, 2003, Vincent *et al.*, 2006).

1.1.3. Morbidity and Mortality

Sepsis is not only the leading cause of death in non-coronary ICUs but also one of the most common causes of morbidity and mortality for all hospitalised patients (Alberti *et al.*, 2003, Martin *et al.*, 2003). Deaths related to severe sepsis exceed the number of persons with other diseases which are more likely to catch the public attention, such as breast cancer and AIDS (Moss, 2005). Angus *et al.* (2001) and Martin *et al.* (2003)

reported that the mortality rate in the United States due to sepsis was approximately 30%, whereas Vincent and colleagues (2006) European ICU study show that the overall ICU and hospital mortality rates were 18.5 and 24.1%, respectively. The EPISEPSIS study group conducted a nationwide survey of patients with severe sepsis in 206 French ICUs over two consecutive weeks (Brun-Buisson *et al.*, 2004). The 30-day mortality rate was 35%. With regards to England, Wales and Northern Ireland, Padkin *et al.* (2003) showed that of the ICU admissions with severe sepsis, 35% died before ICU discharge and 47% died during their hospital stay, whereas Harrison and colleagues (2006) epidemiology study revealed that there was a 45% hospital mortality in 2004. A more recent study showed that in 2010 alone, 5.1% of deaths in England were associated with sepsis (McPherson *et al.*, 2013).

1.1.4. Cost of care

Besides being among the leading causes in critically ill patients, sepsis and septic shock constitute an enormous economic burden. In the United States the annual cost is estimated at $16,7 billion, where the average length of stay and cost per case were 19.6 days and $22,100 (Angus *et al.*, 2001), and between 1997 and 2008, the inflation-adjusted aggregate costs for treating patients hospitalized for this condition increased annually, on average, 11.9% (Hall *et al.*, 2011).

ICU costs relative to patients with septic shock have been analysed by Edbrooke *et al.* in 213 patients in the UK (Edbrooke *et al.*, 1999). The median total cost of care for all septic patients was $10,623, a cost ratio of 6:1 compared with the cost care for non-septic patients of $1,667. The finds of an analysis of 385 patients with severe sepsis from three German ICUs, show that the average costs were €23,297 per patient and €1,318 per day (Moerer *et al.*, 2002). In both the UK and German studies the length of stay and personal costs of septic patients was significantly higher compared to non-septic patients.

1.1.5. Clinical features

The clinical manifestations of sepsis are very unpredictable, depending on the primary site of infection, the causative organism, the pattern of acute organ dysfunction, the patient underlying medical history and the gap before initiation of treatment. The signs of both infection and organ dysfunction can be trivial, therefore Levy and colleagues (2003) provided a list of warning signs of developing sepsis (Table 1.2). This list aims to codify the physical and laboratory findings that aid a clinician to determine whether or not a patient is developing sepsis. Acute organ dysfunction primarily affects the respiratory and cardiovascular systems. Respiratory compromise is manifested as the acute respiratory distress syndrome (ARDS), which is defined as hypoxemia with bilateral infiltrates with no cardiac evidence (Dellinger *et al.*, 2013, Force *et al.*, 2012). Cardiovascular compromise is manifested mostly as hypotension or an elevated blood lactate concentration (Dellinger *et al.*, 2013).

Sepsis (documented or suspected infection plus ≥1 of the following)

General variables

- Fever (core temperature >38.3ºC)
- Hypothermia (core temperature, <36ºC)
- Heart rate (>90 beats per minute or >2 SD above the normal value for age)
- Tachypnea
- Altered mental status
- Significant edema or positive fluid balance (>20ml/kg over 24 hours)
- Hyperglycemia (plasma glucose >120 mg/dl or 7.7mmol/L) in the absence of diabetes

Inflammatory variables

- Leukocytosis (white-cell count >12,000/mm^3)
- Leukopenia (white-cell count <4000/mm^3)
- Normal white-cell count with >10% immature forms
- Plasma C-reactive protein >2 SD above the normal value
- Plasma procalcitonin >2 SD above the normal value

Hemodynamic variables
Arterial hypotention (systolic pressure <90 mmHg, mean arterial pressure <70, or decrease in systolic pressure of >40 mmHg in adults or <2 SD below normal for age).
Mixed venous oxygen saturation >70%
Cardiac index >3.5 litters/min/square meter of body-surface area)
Organ dysfunction variables
Arterial hypoxemia (ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen <300).
Acute oliguria (urine output <0.5ml/kg/hr or 45ml/hr for at least 2 hours)
Creatinine increase >0.5mg/dL
Coagulation abnormalities (international normalized ratio >1.5 or activated partial-thromboplastin time >60 seconds)
Ileus (absent bowel sounds)
Thrombocytopenia (platelet count <100,00/mm^3)
Hyperbilirubinemia (plasma total bilirubin >4mg/dL or 70mmol/L)
Tissue perfusion variables
Hyperlactatemia (>1 mmol/L)
Decreased capillary refill or mottling
Severe sepsis (sepsis plus organ dysfunction)
Septic shock (sepsis plus either hypotention (refractory to intravenous fluids) or hyperlactatemia

Table 1.2: Diagnostic criteria for sepsis, severe sepsis and septic shock. Adapted from Levy *et al.* (2003).

1.1.6. Treatment

Significant improvements in patient outcomes may have resulted from advances in training, better surveillance and monitoring, prompt diagnosis and treatment of the underlying infection. However, the reality is that the case-fatality rates for severe sepsis and septic shock remains very high with those who survive often having impaired physical and neurocognitive functioning, mood disorders, and a low quality of life (Angus *et al.*, 2003).

The development of clinical practice guidelines was produced in order to improve the management of severe sepsis and septic shock. The Surviving Sepsis Campaign Guidelines for Management of Severe Sepsis and Septic Shock were first published in 2004 (Dellinger *et al.*, 2004), revised in 2008 (Dellinger *et al.*, 2008), and revised again and republished in 2013 (Dellinger *et al.*, 2013). The basic elements for the treatment of sepsis and septic shock has not changed much since 1960s: rapid administration of empirical antibiotics that target all likely pathogens, the use of fluid resuscitation and vasopressors to reverse hypotension and maintain tissue perfusion, and if possible, prompt removal or drainage of the source of the infection (Dellinger *et al.*, 2013, Suffredini and Munford, 2011).

Apart from guidelines, sepsis bundles were created in order to ensure all steps of care are consistently delivered. The Surviving Sepsis Campaign and the Institute of Healthcare Improvement worked together to assemble two sepsis bundles, the 3-h resuscitation and 6-h management bundles, which represent a number of treatment goals to be achieved in a set period of time and function as a measurable quality indicators (Figure 1.3). The Surviving Sepsis Campaign guidelines and bundles have led to a sustained and quality improvement in sepsis care, and have been associated with reduced mortality (Levy *et al.*, 2010).

SURVIVING SEPSIS CAMPAIGN BUNDLES

TO BE COMPLETED WITHIN 3 HOURS:
1) Measure lactate level
2) Obtain blood cultures prior to administration of antibiotics
3) Administer broad spectrum antibiotics
4) Administer 30 mL/kg crystalloid for hypotension or lactate ≥4mmol/L

TO BE COMPLETED WITHIN 6 HOURS:
5) Apply vasopressors (for hypotension that does not respond to initial fluid resuscitation) to maintain a mean arterial pressure (MAP) ≥ 65 mm Hg
6) In the event of persistent arterial hypotension despite volume resuscitation (septic shock) or initial lactate ≥4 mmol/L (36 mg/dL):
   - Measure central venous pressure (CVP)*
   - Measure central venous oxygen saturation ($Scvo_2$)*
7) Remeasure lactate if initial lactate was elevated*

*Targets for quantitative resuscitation included in the guidelines are CVP of ≥8 mm Hg, $Scvo_2$ of ≥70%, and normalization of lactate.

Figure 1.3: Surviving Sepsis Campaign 3 and 6 hour bundles. In cooperation with the Institute of Healthcare Improvement the Surviving Sepsis campaign suggested the implementation of a core set of recommendations in order to improve quality in sepsis care. Adapted from Dellinger *et al.* (2013)

1.2. The immune system

The human body is permanently exposed to invasive microorganisms that compromise health and ultimately life. These pathogens seek an opportunity to enter the body through epithelial surfaces such as the skin, respiratory tract, gastro-intestinal tract and genitourinary tract. When these pathogens are successful in entering the body they need to be identified and dealt fast and effectively. This process is performed by the immune system which consists of two distinct, but closely linked processes: innate immunity and adaptive (also known as acquired) immunity.

1.2.1. Innate immune system

The innate immune system provides early host defence against infections. It does so, primarily through anatomical barriers, such as the skin and internal epithelial layers, the peristaltic movement of the gastrointestinal tract and the oscillation of cilia found in the respiratory tract. Additionally, lysozyme found in tears, saliva and nasal secretion act

as antibacterial substances. The more intricate function of the innate immune system involves germline-encoded receptors that detect invading pathogens resulting on the activation of inflammation. This theory was proposed 25 years ago by Charles Janeway, in the now famous paper “Approaching the Asymptote: Revolution and Evolution in Immunology” (Janeway, 1989). In this publication he coined two terms that would revolutionise the way we view the innate immune system. The first was pattern recognition receptors (PRRs), a class of immune-sensor molecules that are capable of recognising infectious agents. The second term was used to describe the ligands they recognise, pathogen-associated molecular patterns (PAMPs). These are highly conserved structures and are recognised by the innate immune system as foreign to the body. PAMPs are essential for the survival and proliferation, so their detection is essential in order to trigger an appropriate immune response.

The detection of infectious agents is made by the so called “trinity of innate sensors” which include Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). The recognition of PAMPs by one of these families of PRRs results in the activation of signalling cascades that induce the secretion of inflammatory cytokines, chemokines and type-I interferons (IFN) in order to eradicate pathogens, stimulate tissue repair and activate an adaptive immune response (Kumar *et al.*, 2011).

1.2.2. Adaptive immune system

Unlike the innate immune system, which is the primary barrier to infectious agents and which acts immediately, the adaptive immune system mediates a delayed response to foreign antigen. This branch of the immune system is crucial for the control of pathogens that escape removal by the innate immune response and essential in the development of immunological memory. This procedure operates via generation of antigen specific B and T-cell receptors through a process of gene rearrangement which have specificity for a PAMP expressed by the invading microorganism. One of the most

cunning methods the adaptive immune system has towards the eradication of pathogens is the production of plasma cells and memory cells. When a B-cell comes into contact with an antigen it proliferates and generates plasma cells, which release antibodies relevant to the antigen recognised in order clear it, whilst memory cells are produced in order to prepare the body for future challenges of the same pathogen. If the body is exposed to a second challenge of the same pathogen, the immune response is much faster compared to the initial challenge and consequently the eradication of the infectious agent is also much faster (Hoebe *et al.*, 2004, Iwasaki and Medzhitov, 2010).

1.2.3. Dysfunctional immune responses

Under normal circumstances the immune system can distinguish self from foreign molecular components. Many of the diseases that afflict mankind are a consequence of a failure of this precise mechanism, where a dysfunctional innate and/or adaptive immune system recognises self antigens as foreign. This is referred to as an autoimmune disease and both the innate and adaptive immunity are now seen as accountable for this detrimental outcome. Septic shock is the purest example of host injury mediated by a systemic innate immune activation. Other examples include rheumatoid arthritis and systemic lupus erythematosus which are also correlated with the over-production of inflammatory cytokines (TNF-α and type-I IFN, respectively) (Beutler, 2004, Hoebe *et al.*, 2004).

1.3. Pattern recognition receptors

Germ-line encoded pattern-recognition receptors (PRRs) of the innate immune system can be divided into three distinct groups: Toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat-containing (NBD-LRR) proteins (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs).

1.3.1. Toll-like receptors

Toll-like receptors (TLRs) are the most widely studied PRRs and considered to be essential in pathogen recognition. There are thirteen mammalian TLRs, ten of which are found in humans and twelve of which are found in mice. TLRs were first identified in 1985 by Dr. Nüsslein-Volhard and co-workers, when they screened *Drosophila melanogaster* for embryonic polarity genes. They found a receptor that was involved in the production of the dorsal-ventral axis in embryos, and dubbed it Toll. Developing *Drosophila* embryos could not establish a proper dorsal-ventral pattern and appeared "toll" ("weird" in German colloquial speech) (Anderson *et al.*, 1985). In 1991, Gay *et al.* found that the cytoplasmic domain of Toll was related to that of the human interlukin-1 receptor (IL-1R). The amino acid sequence of the Toll receptor in the *Drosophila* and the IL-1R was identical throughout most of the domain, where 135 amino acids were identical. In this publication, the extracytoplasmic section of the Toll protein, made of leucine-rich repeat proteins (LRRs) was also described in this publication (Gay and Keith, 1991). The link between Toll and its pivotal role in triggering an immune response was only made in 1996. Lemaitre and colleagues found that Toll-deficient adult *Drosophila* infected with *Aspergillus fumigates*, a pathogen for insects, died after 2-3 days - half the time compared to the wild-type population. This study demonstrated that Toll was essential for *Drosophila* to mount an immune response against the fungus (Lemaitre *et al.*, 1996).

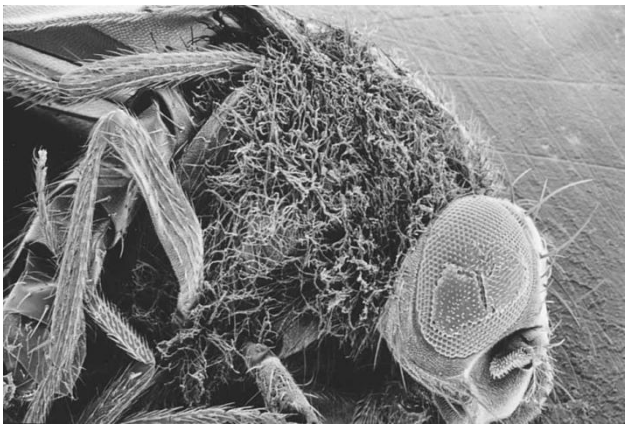


Figure 1.4: Scanning electron micrograph of germinating hyphes of *Aspergillus fumigates* on a dead *Drosophila melanogaster* adult (200x magnification) (Lemaitre *et al.*, 1996).

In 1997, Medzhitov and Janeway cloned and characterized the first human Toll homologue, hToll, which shared homology with the *Drosophila* Toll (dToll) over the entire length of the protein chains (Medzhitov *et al.*, 1997). A year later, there were two major discoveries in the TLR field. Firstly, Rock *et al.* coined the term Toll-like receptors (TLRs) and described 5 homologues of Toll (TLRs 1-5) (Rock *et al.*, 1998). Secondly, Poltorak *et al.* discovered that TLR4 is the receptor for bacterial lipopolysaccharide (LPS), a component found on the outer membrane of Gram-negative bacteria, which finally cemented the idea of TLRs being able to recognise infectious agents (Poltorak *et al.*, 1998). The discovery of the remaining known TLRs came in quick succession. Takeuchi *et al.* (1999) discovered TLR6, followed by TLRs 7, 8, 9 (in 2000) and 10 (in 2001) (Chuang and Ulevitch, 2001, Chuang and Ulevitch, 2000).

1.3.1.1. Toll-like receptor structure

TLRs are type I membrane glycoproteins, which possess three domains: an extracellular leucine rich repeats (LRRs), a transmembrane domain and a cytoplasmic TIR domain (Figure 1.5). The extracellular domain of the TLR family proteins contain between 16 and 28 LRRs and they all share a common structural motif

“xLxxLxxLxLxx”, 20-30 amino acids long. This domain contains a β-strand and an α-helix linked by hoops, which are thought to grant the LRR a unique horseshoe architecture. The LRRs are thought to create the binding site for PAMPs. The cytoplasmic domain of TLRs is called Toll/IL-1 receptor (TIR) domain due to its similarity to the IL-1 receptor. This domain consists of approximately 200 amino acids. Within the TIR domain there are three highly conserved regions (Box 1, 2 and 3) which are required for the interaction and recruitment of intracellular proteins that participate in the signalling pathways of TLRs (Carpenter and O'Neill, 2009, Jin and Lee, 2008, Kumar *et al.*, 2011).

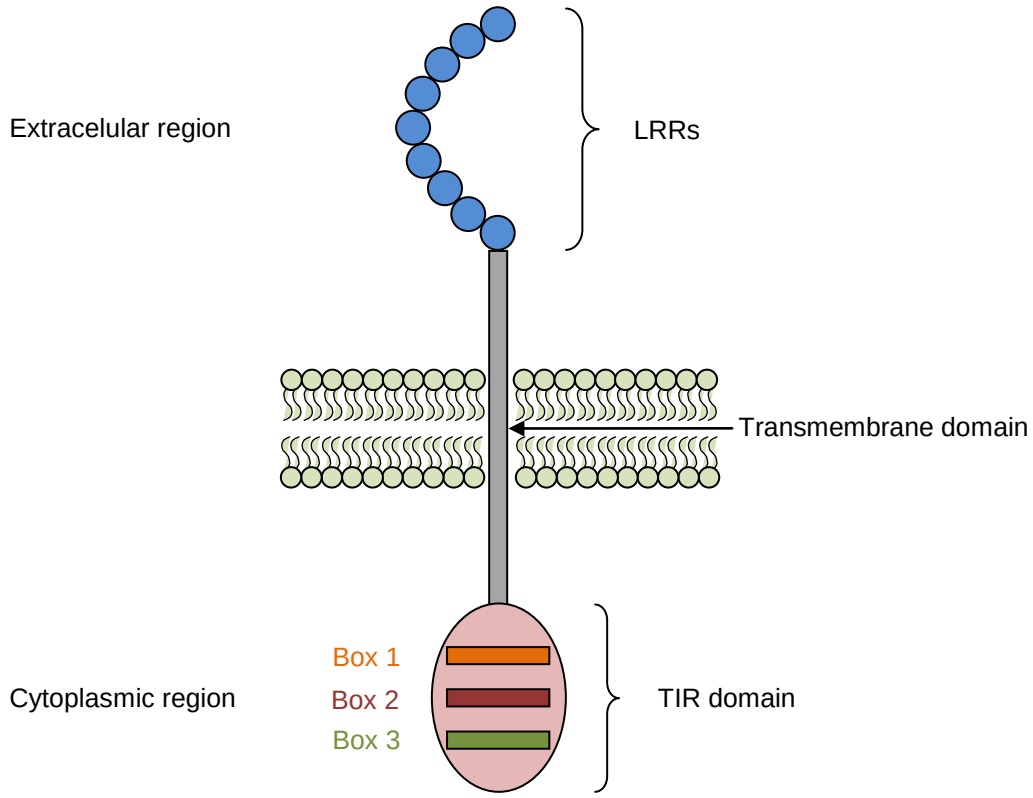


Figure 1.5: Toll-like receptor family basic structure. The extracellular domain consists of 16-28 leucein rich repeats (LRRs) at the N-terminus, followed by a transmembrane domain. The cytoplasmic domain consists of the Toll/IL-1 receptor (TIR) domain at the C-terminus. The LRRs form a horseshoe-like structure that creates the binding site for PAMP. The TIR domain possesses three highly conserved regions: box 1 is considered the signature sequence of the family, whereas box 2 and 3 contain amino acids critical for signalling.

1.3.1.2. Individual Toll-like receptors

So far, ten functional TLRs have been discovered in humans and twelve functional TLRs have been found in mice. TLR1-9 are conserved in both human and mice but TLR10 is not functional in mice. This is due to a retrovirus insertion, which substituted the C-terminal half of the receptor for an unrelated and non-productive sequence in the *Tlr10* gene. Mice do express TLR11, TLR12 and TLR13, but these have been lost in the human genome, with the *Tlr11* gene having a stop codon that results in a lack of production of the receptor. The function of TLR10 in humans, and TLR12 and TLR13 in mice remains unknown (Beutler, 2004, Kawai and Akira, 2009, Kawai and Akira, 2010, Kumar *et al.*, 2011).

TLR1, 2, 4, 5, 6 and 11 are all present on the cell surface, with their LRR domain facing out of the cell, whereas TLR3, 7, 8 and 9 are exclusively expressed within the endosomal membrane, with their LRR domain facing into the endosomal compartment. Most TLRs are believed to be homodimers, although heterodimers do exist in the case of TLR2/TLR1 and TLR2/TLR6. Once a PAMP binds to its respective TLR it recruits a single or a combination of TIR-domain-containing adaptor proteins. This results in the activation of the signalling cascade, which in turn results in the production of proinflammatory cytokines as well as type-I interferon (Kawai and Akira, 2011).

1.3.1.2.1. Cell surface TLRs and their ligands

TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface and recognise microbial membrane components such as lipids, lipoproteins and proteins.

1.3.1.2.1.1. TLR1, 2 and 6

TLR2 is capable of forming stable heterodimers with TLR1 and TLR6 or non-TLR molecules such as CD14, CD36 and dectin-1. This confers the ability of TLR2, in conjunction with bound heterodimers, to recognize a wide range of PAMPs from

bacteria, parasites, fungi and viruses. These include lipoproteins and lipopeptides, lipoteichoic acid (LTA) and peptidoglycan from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi and viral components such as haemaglutinin protein from the measles virus (Akira *et al.*, 2006, Kawai and Akira, 2011). In particular, the TLR2/TLR1 heterodimer recognises triacylated lipopeptides as its main PAMP, and on the other hand, the TLR2/TLR6 heterodimer recognises diacylated lipopeptides (Kawai and Akira, 2010).

In 2007, the crystal structure of the human TLR2/TLR1 heterodimer was discovered. TLR2 forms a stable heterodimer with TLR1 which, with the addition of the triacylated lipopeptide, Pam_3CSK_4, shows that the overall shape of the complex resembles the letter “m” (Figure 1.6). The formation of the TLR2/TLR1/Pam_3CSK_4 heterodimer allows the initiation of downstream signalling (Jin *et al.*, 2007).

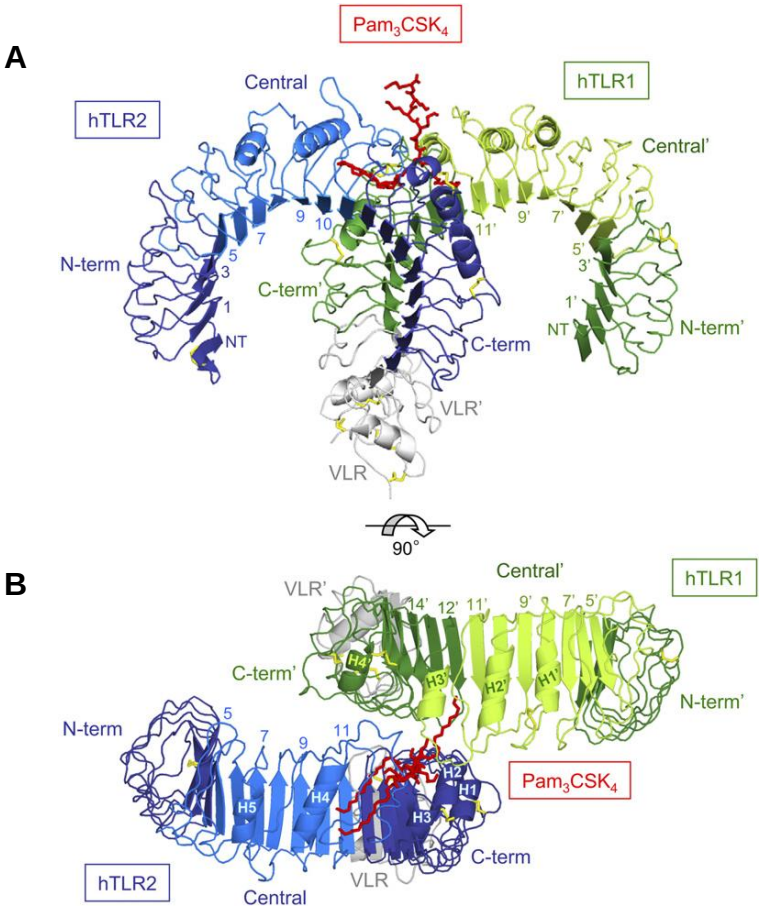


Figure 1.6: The structure of the human TLR2/TLR1/Pam_3CSK_4. This structure shows that two of the three hydrophobic lipid chains on Pam_3CSK_4 (red) are submerged in the hydrophobic spaces within TLR2 (blue), with the remaining hydrophobic lipid chain within TLR1 (blue). TLR2/TLR1/Pam_3CSK_4 forms an "m" shaped heterodimer. A) side view; B) top view. (Jin *et al.*, 2007).

In 2009, Kang *et al.* determined the crystal structure of TLR2/TLR6 heterodimer. Similar to TLR1, TLR6 also forms an "m"-shaped heterodimer with TLR2 with the addition of the diacyl lipopeptide, Pam_2CSK_4 (Figure 1.7.). The stability of TLR2/TLR1/Pam_3CSK_4 complex relies upon the interaction between the amide-bound lipid and TLR1. On the other hand, in the TLR2/TLR6/Pam_2CSK_4 complex, the bridging role of the amide lipid chain is missing. Nevertheless, TLR2 and TLR6 are able to form a stable heterodimer due to the increased hydrophobic interactions between the two TLRs. These interactions seem to compensate for the reduced lipid chain interaction (Kang and Lee, 2011, Kang *et al.*, 2009).

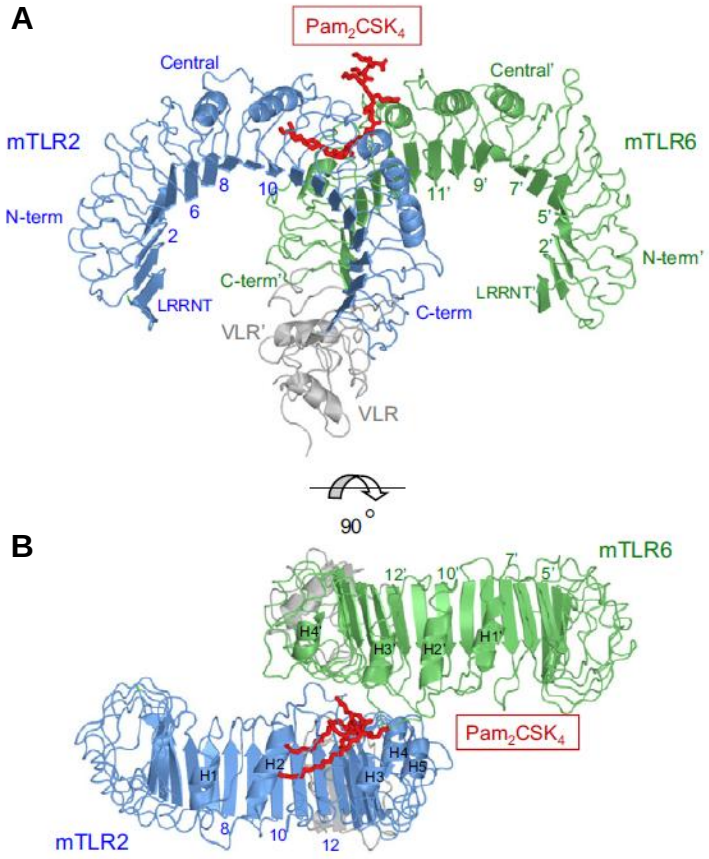


Figure 1.7: The structure of the mouse TLR2/TLR6/Pam_2CSK_4 complex. This structure shows the two esther-bound lipids on Pam_2CSK_4 (red) are inserted into hydrophobic pockets within TLR2 (light blue) but not within TLR6 (light green). TLR2 and TLR6 can still form stable heterodimers because the increased hydrophobic interaction between the two TLRs. TLR2/TLR6/Pam_2CSK_4 forms an "m" shaped heterodimer. A) side view; B) top view. (Kang *et al.*, 2009).

1.3.1.2.1.2. TLR4

TLR4, a founding member of the TLR family, is responsible in the recognition of bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. TLR4 detects the presence of LPS through the formation of a trimolecular receptor complex, involving CD14 and myeloid differentiation factor 2 (MD-2). LPS-binding protein (LBP), a soluble protein, is the first protein to encounter circulating LPS. LPS and LBP bind in the serum and form the LPS-LBP complex, which

in turn bind to CD14 on the cell surface (Tobias *et al.*, 1997). Subsequently, CD14, a glycosylphosphatidylinositol (GPI)-linked protein, delivers the LPS-LBP to the TLR4-MD-2 complex (Akashi *et al.*, 2000, Shimazu *et al.*, 1999). In response to LPS, TLR4 has been shown to associate with other receptor clusters such as heat shock proteins (Hsps), in particular Hsp70 and Hsp90; chemokine receptor (CXCR4) and leukocyte integrins CD11b/CD18 (Triantafilou and Triantafilou, 2004).

TLR4 has also been shown to be involved in the recognition of respiratory syncytial virus (RSV) fusion proteins, mouse mammary tumour virus and *Streptococcus pneumoniae* pneumolysin (Burzyn *et al.*, 2004, Kawai and Akira, 2010).

The crystal structure of mouse TLR4 has been reported. Two studies, one in 2007 and one in 2009, published the crystal structure of mouse TLR4-MD-2 heterodimer bound to Eritoran (an antagonist PAMP) or lipid A (an agonist PAMP). The structural study of TLR4-MD-2 in complex with LPS has shown that four of four acyl chains of Eritoran and five of the six acyl chains of lipid A are submerged within the hydrophobic space of MD-2. The remaining lipid chain of lipid A is inserted into the hydrophobic space of TLR4. This aids the interaction between MD-2 and TLR4, resulting in the activation of downstream signalling for lipid A, unlike Eritoran, as it has no direct interaction with TLR4 (Kim *et al.*, 2007, Park *et al.*, 2009).

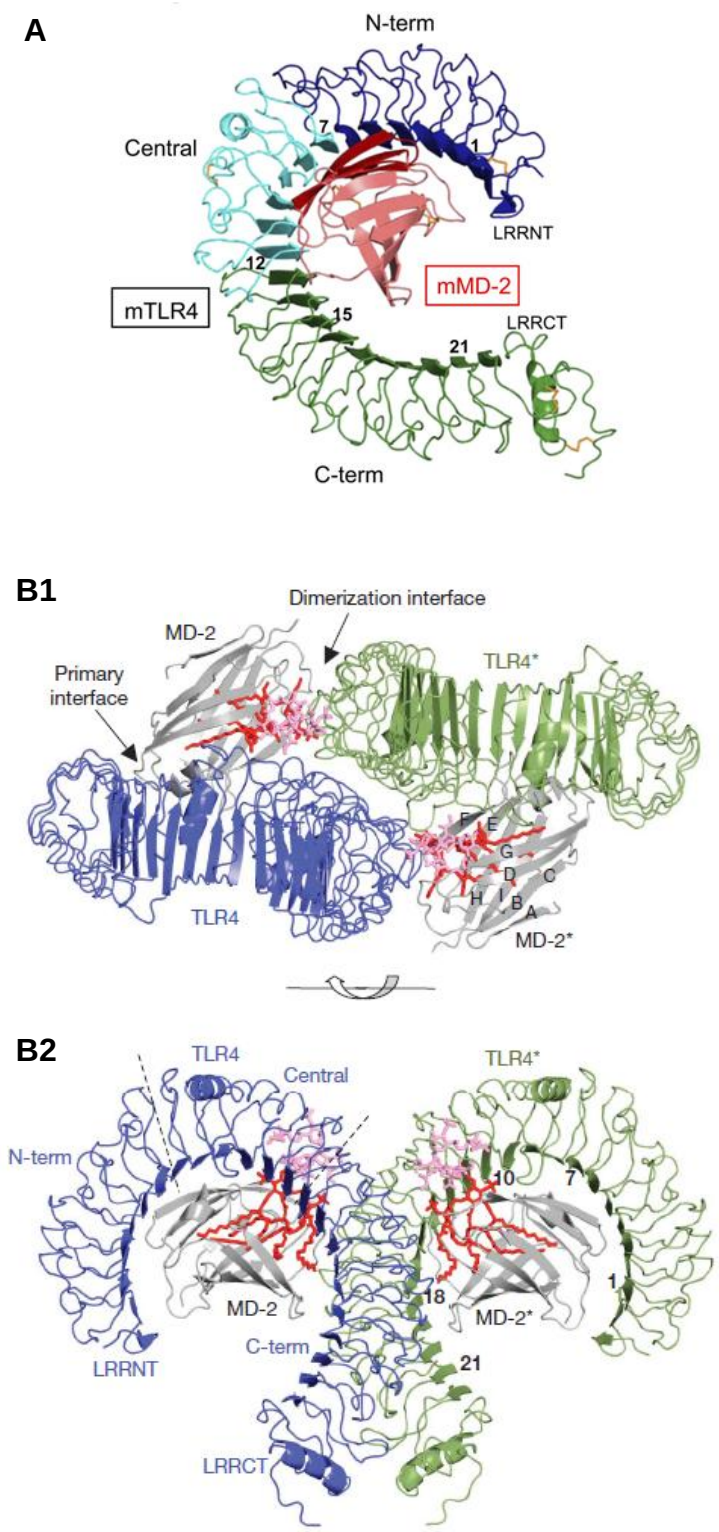


Figure 1.8: Structure of the TLR4-MD-2 complex. A) Structure of the mouse TLR4-MD2 complex. The N-terminal, central, and C-terminal domains of TLR4 are coloured blue, cyan and green, respectively. MD-2 is shown in pink and red (Kim *et al.*, 2007). B1) and B2) Top and side view, respectively, of the TLR4-MD-2-LPS homodimer complex. Lipid A component of LPS is shown in red and pink, MD-2 in grey and the TLR4 receptors in blue and green. Adapted from Park *et al.* (2009).

1.3.1.2.1.3. TLR5 and mouse TLR11

TLR5 is responsible for sensing flagellin, a protein expressed by flagellated bacteria (Hayashi *et al.*, 2001). TLR5 has been shown to be expressed on the intestinal epithelial cells, and endothelial cells and also highly expressed in $CD11c^{+}CD11b^{+}$ lamina propria DCs in the small intestine (Uematsu *et al.*, 2008).

Mouse TLR11 is thought to be a close relative to TLR5, and is highly expressed in the bladder and kidneys. In 2004, Zang and colleagues showed that TLR11-deficient mice are prone to infection with uropathological bacterial and concluded that TLR11 plays an important role in preventing infections of the urogenital system. Human TLR11 non-functional duo to a stop codon in the gene (Zhang *et al.*, 2004).

1.3.1.2.1.4. TLR10

TLR10 is expressed in humans but not in mice, but little is known about the ligand and cascade activated by this TLR.

1.3.1.2.2. Intracellular TLRs and their ligands

TLR3, TLR7, TLR8 and TLR9 are localised in intracellular vesicles such as the endosomes membrane and the endoplasmic reticulum (ER). These endosomal TLRs predominantly recognise pathogenic nucleic acids, DNA, single-stranded-DNA (ssDNA), single- and double-stranded-RNA (ssRNA and dsRNA, respectively).

1.3.1.2.2.1. TLR3

TLR3 is involved in the recognition of double-stranded ribonucleic acid (dsRNA). In 2001, experiments with TLR3-deficient mice showed that they had reduced responses to a synthetic analogue of dsRNA, polyinosinic-polycytidylic acid (poly(I:C)), resulting in a lower secretion of inflammatory cytokines (Alexopoulou *et al.*, 2001). TLR3 can also

recognise single-stranded (ssRNA) produced during the replication of dsRNA viruses such as RSV and the West Nile virus (Groskreutz *et al.*, 2006, Wang *et al.*, 2004).

Leonard *et al.* showed in a biochemical study that dsRNA, but not dsDNA or ssRNA, induces homodimerization of TLR3 (Leonard *et al.*, 2008). Liu and co-workers determined the crystal structure of TLR3 bound to a dsRNA ligand. The architecture of the TLR3 homodimer, induced by the binding of dsRNA, is similar to the “m” shape dimers of the TLR2 and TLR4 complexes (Figure 1.9). Although it resembles the overall structure, the TLR3 homodimer has a different mechanism for recognising the ligand of the TLR4-MD-2, TLR2/TLR1 and TLR2/TLR6 complexes. Both the C- and N-terminal on the lateral side of the convex surface of each TL3 dimer interact with dsRNA ligand (Kang and Lee, 2011, Liu *et al.*, 2008).

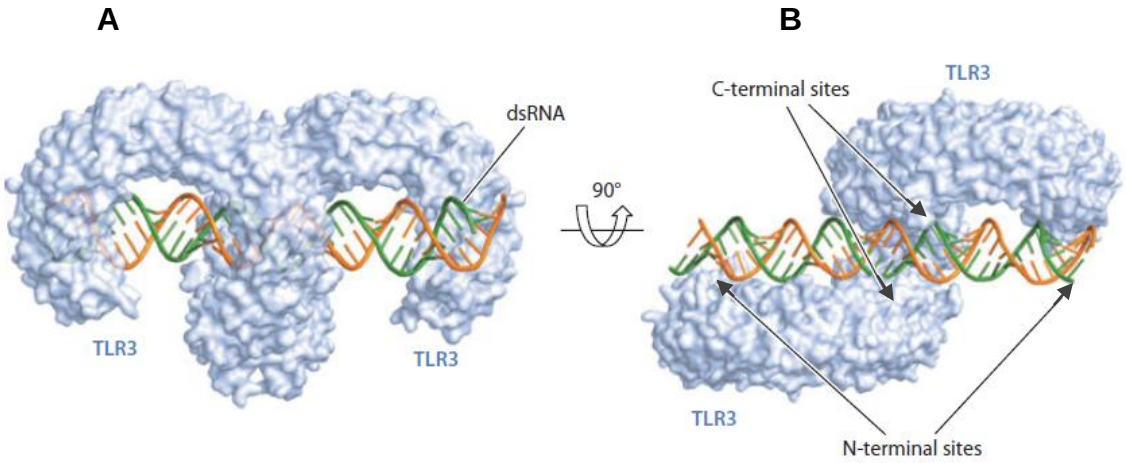


Figure 1.9: Structure of the TLR3 homodimer associated with double-stranded RNA (dsRNA). TLR3 homodimer in light blue, dsRNA in green and orange. The C- and N-terminal RNA interaction sites with TLR3 are marked in arrows. A) side view; B) top view. Adapted from (Kang and Lee, 2011).

1.3.1.2.2.2. TLR7 and TLR8

TLR7 and TLR8 are phylogenetically very similar and are responsible for the detection of viral ssRNA. These receptors were originally proposed to be responsible in sensing imidaziquiniline (imiquimod and resiquimod (R-848)) and guanine analogs. Murine experiments showed that TLR7-deficient mice did not show any inflammatory cytokine production when challenged with imidazoquinolines (Hemmi *et al.*, 2002). TLR7 and TLR8 can also recognise ssRNA from RNA viruses including influenza A virus and human immunodeficiency virus (HIV) (Kawai and Akira, 2009).

1.3.1.2.2.3. TLR9

TLR9 detects foreign deoxyribonucleic acid (DNA). In 2000, Hemmi *et al.* showed that TLR9 knock-out mice were unable to detect 2'-deoxyribo(cytidine-phospate-guanosine) (CpG) DNA motifs that are found in DNA bacteria and DNA viruses (Hemmi *et al.*, 2000). The sugar back-bone of DNA (2'-deoxyribose) is enough to bind and activate TLR9. This is important to distinguish between "self" DNA, when it has a phosphodiester (PD) backbone, and unnatural, or foreign DNA, when it has a phorothionate (PS) backbone (Haas *et al.*, 2008). TLR9 has also been linked to detect crystal hemozoin from parasites (Coban *et al.*, 2010).

1.3.1.3. TLR ligands

TLRs are one of the most important PRRs groups discovered to date. These receptors homo and hetero-dimerize in order to recognise a broad spectrum of PAMPs derived from microbial pathogens such as viruses, bacteria, fungi and parasites (Table 1.3). This broad recognition of PAMPs by the different TLRs triggers the release of inflammatory cytokines that orchestrate a rapid and effective pathogen removal. TLRs 1, 2, 4, 5, 6 and 11 are cell surface bound whilst TLRs 3, 7, 8, 9 are located in the endosomal membrane.

Receptor	Ligand	Source of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phospholipomannan GPI-mucin Envelope glycoproteins Porins Zymosan B-Glycan Heat-shock protein 70	Various pathogens Gram-positive bacteria Gram-positive bactéria Mycobacteria *Candida albicans* Protozoa Viruses (e.g., HSV, measles virus) *Neisseria* Fungi Fungi Host
TLR3	dsRNA	Viruses
TLR4	Lipopolysaccharide (LPS) Mannan Fusion proteins Envelope glycoproteins Glycoinositolphospholipids Heat-shock protein 60, 70 Fibrinogen	Gram-negative bacteria *Candida* RSV Mammary-tumor virus *Trypanosoma* Host Host
TLR5	Flagellin	Flagellated bacteria
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	*Mycoplasma* Gram-positive bacteria Fungi
TLR7	ssRNA Imidazoquinoline	RNA viruses Synthetic compounds
TLR8	ssRNA Imidazoquinoline	RNA viruses Synthetic compounds
TLR9	CpG motifs Hemozoin	Bacteria and Viruses *Plasmodium falcuparum*

hTLR10	Not determined	Not determined
mTLR11	Not determined Profilin-like molecule	Uropathogenic bacteria *Toxoplasma gondii*
mTLR12	Not determined	Not determined
mTLR13	Not determined	Not determined

Table 1.3: Toll-like receptors, the ligands they recognise and their source (Akira *et al.*, 2006, Kawai and Akira, 2009, Kawai and Akira, 2011, Kumar *et al.*, 2011, Mogensen, 2009).

1.3.1.3.1. Lipopolysaccharide

Bacteria are unicellular prokaryote microorganisms consisting of many members of various shapes and sizes. Although diverse, all PAMPs from bacteria can be recognised by the different TLRs. Lipopolysaccharide (LPS) is an important structural component found on the outer membrane of Gram-negative bacteria which consists of a hydrophobic domain known as lipid A (endotoxin), a non-repeating core oligosaccharide region, and a distal O-specific chain (O-antigen) polysaccharide (Figure 1.10) (Beutler and Rietschel, 2003). Lipid A, the hydrophobic anchor of LPS, is a glucosamine-based phospholipid that constitutes the outer monolayer of the outer membranes of Gram-negative bacteria. In a single *E. coli* cell there are around 10^6 lipid A residues, and because they have a conserved architecture, they are detected at picomolar levels by TLR4 (Galloway and Raetz, 1990). The hydrophilic polysaccharide unit is composed by a core region and an O-specific chain. The core oligosaccharides contains an inner (lipid A proximal) and an outer core. The outer core provides an attachment site for the O-specific chain, whereas the inner core contains residues of Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) and L-*glycero*-D-*manno*heptose (L,D-Hep)

oligosaccharide and can contain phosphates, amino acids and sugars such as 2-keto-3-deoxyoctulsonic acid. In comparison to the core region, the O-specific chain is structurally more variable, which gives rise to the different Gram-negative bacterial strains. For example, in *E. coli* strains there are around 170 different O-specific chain structures (Raetz and Whitfield, 2002).

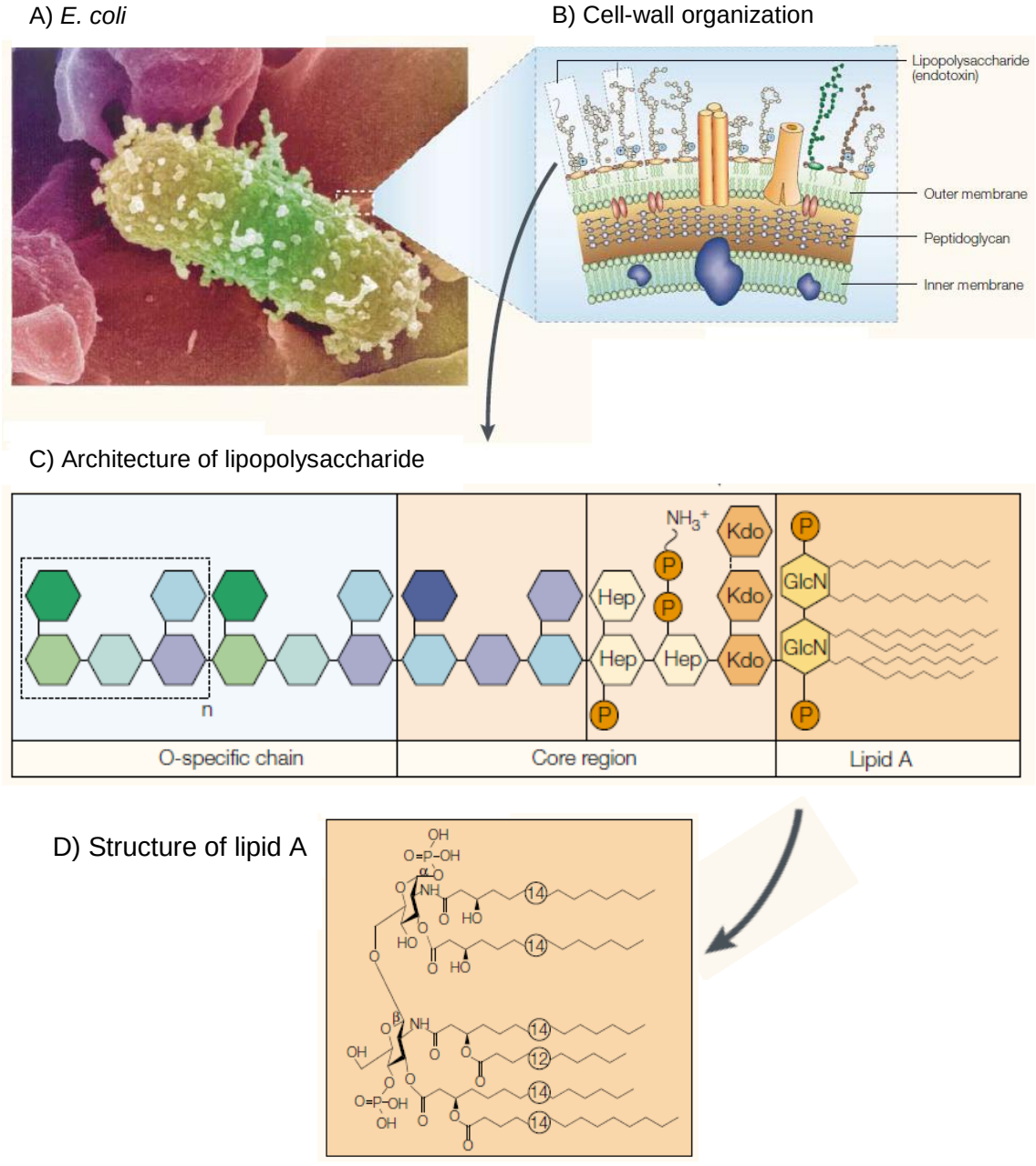


Figure 1.10: A Gram-negative cell wall. A) electron micrograph of *E. coli*; B) location of lipopolysaccharide (LPS), outer membrane, peptidoglycan and inner membrane in the bacteria cell wall; C) schematic diagram of the toxic centre of the Gram-negative lipopolysaccharide (LPS); D) the lipid A component. GlcN: D-glucosamine; Hep: L-glycero-D-manno-heptose; Kdo: 2-keto-3-deoxy-octulosonic acid; P: phosphate. Adapted from Beutler and Rietschel (2003).

1.3.1.4. CD14

Even though TLRs are the key detectors of invading pathogens, they require adapter molecules for the effective activation in response to the different PAMPs. The efficacy of TLR4 activation is greatly enhanced by CD14, a 55 kDa glycosylphosphatidylinositol (GPI)-anchored protein, and one of the first adapter molecules to be identified as a co-receptor for LPS (Wright *et al.*, 1990).

The crystal structure of CD14 has been determined to a 2.5 Å resolution. It was shown that the CD14 monomer contains thirteen β strands where eleven of them (β3-13) overlap with conserved leucine-rich repeats (LRRs). CD14 exists as a dimer in solution as well as in the crystallographic asymmetric unit (Figure 1.11). Its horseshoe-like structure and the number of conserved LRRs is close to those in TLR4, which shows close similarities with between the adaptor molecule and the TLR family (Kim *et al.*, 2005).

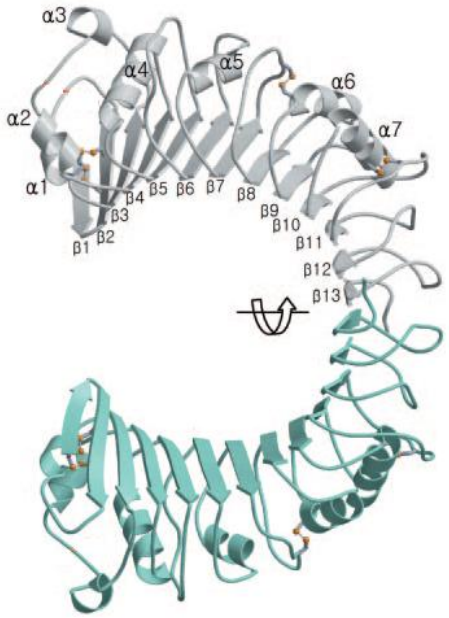


Figure 1.11: Overall structure of the CD14 dimer. Two monomers of CD14 in the crystal are coloured gray and cyan. Disulfide bridges are shown in orange. Adapted from Kim *et al.* (2005).

The main role of CD14 in LPS recognition was demonstrated by Haziot *et al.* (1996) in CD14-knockout mice. The group demonstrated that CD14-deficient mice were 10,000-fold less sensitive to LPS compared to control mice.

The recognition of LPS through TLR4 involves a series of steps which start from the binding of LPS by LPS-binding protein (LBP) which transfers the bound LPS to CD14, which in turn facilitates the transfer of LPS to the TLR4 which activates the signalling pathway (Kawai and Akira, 2011).

1.3.1.5. TLR signalling

As soon as a particular PAMP has been recognised by its respective TLR, it dimerises, often forming homodimers or, in the case of TLR1, 2 and 6, a heterodimer. The TLR dimer undergoes a conformational change which results in the recruitment and activation of intracellular TIR domains, which activates downstream signalling, which subsequently activates transcription factors. These transcription factors are then translocated to the nucleus where they cause an up-regulation of genes that are responsible for the inflammatory and antiviral response.

TLR signalling is divided into two different pathways: the myeloid differentiation primary gene-88 (MyD88)-dependant and MyD88-independent pathway (Figure 1.12). MyD88 adaptor molecule is utilised by all the TLRs, except TLR3, in order to induce downstream signalling. TLR3 utilises a second pathway, the MyD88-independent pathway, i.e. the TRIF-dependent pathway. TLR4 can signal in a MyD88-dependant and independent manner. TLR signalling requires other adaptor molecules which accurately transduce the signal, including four in particular: MyD88, TIR domain-containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) and TIR-domain containing adaptor protein (TIRAP, also known as MAL (MyD88-adaptor like)). These adaptor molecules, like all the TLRs, contain and interact through a Toll-interleukin 1 receptor (IL-1R) homology domain (TIR domain) (Kawai

and Akira, 2010). MyD88, is used by all the TLRs, with the exception of TLR3, and activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) in order to produce inflammatory cytokines. On the other pathway, TRIF is used by TLR3 and TLR4 in order to activate the transcription factors NF-κB and interferon regulatory factor 3 (IRF3), which consequently leads to the production of inflammatory cytokines and type-I interferon. The adaptor molecules TRAM and TIRAP recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. Consequently, TLR downstream signalling can be categorised as either MyD88-dependent, responsible for the production of inflammatory cytokines, or MyD88-independent (TRIF-dependent) pathways, which induces the production of both type-I IFN and inflammatory cytokines (Kawai and Akira, 2011).

1.3.1.5.1. MyD88-dependent pathway

After MyD88 is activated by TLRs it recruits the IL-1R-associated kinase (IRAK) family protein kinases IRAK4, IRAK1, IRAK2 and IRAKM. MyD88 recruits IRAK4 through a death domain (DD)-DD association, which in turn associates with IRAK1 and IRAK2 causing IRAK-1 phosphorylation and activation. This results in the interaction of IRAK1 with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), forming the IRAK-1-TRAF-6 complex (Kawagoe *et al.*, 2008). IRAKMs main function is to block the formation of this complex (Kobayashi *et al.*, 2002). IRAK1 bound to TRAF6 dissociates from the complex and binds with transforming growth factor-B-activated kinase 1 (TAK1) and the TAK1 binding proteins 2 and 3 (TAB2 and TAB3). This complex activates TAK1, which phosphorylates the IκB-kinase (IKK) complex which is made up of NEMO (NF-κB essential modulator, also known as IKK-γ) IKK-α and IKK-β. IκB is then phosphorylated and undergoes proteosomal degradation, causing the release of NF-κB which can then translocate to the nucleus where it induces gene transcription of proinflammatory cytokines (Kawai and Akira, 2009, Kawai and Akira, 2010, Kumar *et al.*, 2011).

1.3.1.5.2. MyD88-independent pathway

The MyD88-independent, also called TRIF-dependent pathway, induces the expression of type-I IFN and is activated only by TLR3 or TLR4. TRIF is bound to TLR3 through its TIR domain, whereas TLR4 uses TRAM, which is associated with TRIF, in order to activate this signalling pathway. Following this, TRIF associates with TRAF3 and activates the IKK complex of TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε. This complex mediates direct phosphorylation of IFN regulatory factor 3 (IRF3) and IRF7. This phosphorylation causes IRF3 and IRF7 to form hetero- or homodimers, translocate to the nucleus and with the aid of coactivators such as CBP and p300, initiate transcription of IFN and IFN-inducible genes in order to induce type-I IFN expression. TRIF-dependent pathway can also activate NF-κB. This occurs when the TLR4 TRIF recruits TRAF6 as well as receptor-interacting protein 1 (RIP-1) and TNF receptor (TNFR)-associated death domain (TRADD), which forms a complex in conjunction with TRAF6 after RIP-1 is polyubiquinated. These two molecules activate TAK1, which consequently activate the NF-κB pathway (described previously in 1.3.5.1) (Kawai and Akira, 2010, Mogensen, 2009).

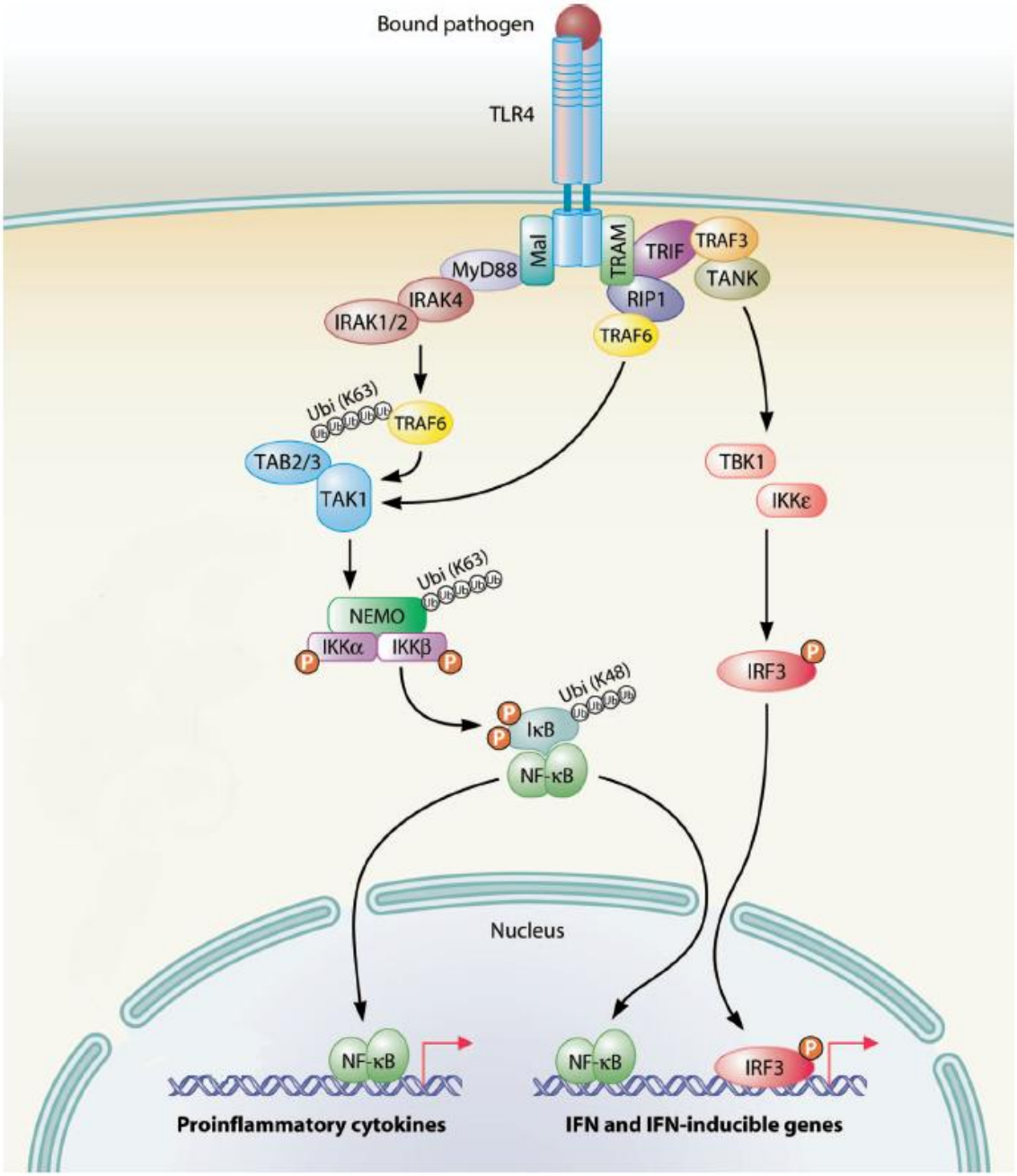


Figure 1.12. Toll-like receptor signalling pathways. TLR4 is able to activate both MyD88-dependent (shown on the left) and – independent (TRIF-dependent) (shown on the right) pathways. The MyD88-dependent pathway is responsible for the activation of NF-κB which consequently controls the induction of proinflammatory cytokines. On the other hand, the MyD-88-independent pathway activates IRF3/7 in order to induce type-I IFN expression. Adapted from (Mogensen, 2009).

1.3.2. NOD-like receptors

Nod-like receptors (NLRs) form part of the 'trinity of pathogens sensors' - a term coined by Creagh and O'Neill (2006), combined with the TLRs and RLRs. There are 22 different NLRs members known in the human genome and many more in mice. NLRs are located in the cytosol and share a unique domain architecture consisting of a central nucleotide-binding and oligomerisation (NACHT) domain, which is located between C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. The LRR domain is thought to be the ligand binding domain as well as having an autoregulation, whereas CARD and PYD domains are involved in homotypic protein-protein interactions necessary for downstream signalling (Figure 1.14). The N-terminal domain has been used to separate the NLR family into three subgroups: the nucleotide-binding oligomerisation domain (NOD) (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1 and CIITA); the NLRPs (NLRP1-14, also known as NALPs); and NLRC4 (also known as IPAF) (Schroder and Tschopp, 2010, Stutz *et al.*, 2009). Functionally NLRs can be separated on their ability to form a complex known as the inflammasome.

1.3.2.1. Inflammasome

The term inflammasome was first used in 2002 by Martinon and colleagues, due to its similarity to the apoptosome and its ability to cause inflammation. The inflammasomes are a group of multimeric protein complexes that consist of an activated receptor molecule, apoptosis-associated speck-like protein containing CARD (ASC) and pro-caspase-1. The formation of the inflammasome complex is triggered by a variety of substances derived from infections, tissue damage or metabolic dysfunctions (Martinon *et al.*, 2002). Ultimately, the arrangement of the inflammasome complex triggers the activation of pro-caspase-1, which proteolytically activates the pro-inflammatory cytokines IL-1β and IL-18.

The inflammasomes are arranged with an inflammasome sensor molecule that is connected to pro-caspase-1 via ASC. ASC is common to all inflammasomes and it contains two death fold domains: one pyrin (PYD) and one CARD domain. In their inactive state, NLR is kept inactive and once it binds to its specific ligand, a conformational change occurs which enables the NLR to bind to ASC through a PYD-PYD homotypic interaction. This interaction triggers ASC to bring monomers of pro-caspase-1 into close proximity, via CARD-CARD homotypic interactions, which initiates the activation of caspase-1 self-cleavage and the formation its active form. The main role of caspase-1 is to activate pro-IL-1β and pro-IL-18. This is the second step in the conversion of pro-IL-1β into its bioactive cytokine IL-1β, as it requires a priming signal. This signal is induced by the activation of NF-κB, which is promoted by TLR recognition of their ligands. On the other hand, pro-IL-18 does not need a priming signal as it is constitutively expressed and its expression is increased after cellular activation (Davis *et al.*, 2011, Latz *et al.*, 2013, Stutz *et al.*, 2009).

A number of inflammasome sensor molecules can activate caspase-1 and lead to IL-1β production, suggesting roles in inflammasome formation. These include NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7 NLRP12, NLRC4 (NOD-, LRR- and CARD-containing 4, or IPAF), NOD2. Two other non-NLRs have been described as inducing the formation of inflammasome: the PYTHIN (pyrin and HIN domain-containing protein) family members absent in melanoma 2 (AIM2) and IFN-γ-inducible protein 16 (IFI16) (Hornung and Latz, 2010, Latz *et al.*, 2013). Although for some of these NLRs it is only suggested that they form inflammasomes, others, such as NLRP1 and NLPR3 have been widely documented to form inflammasomes.

1.3.2.1.1. NLPR1 inflammasome

From the NLR family, NLRP1 was the first to be shown to form an inflammasome (Martinon *et al.*, 2002). NLRP1 contains an N-terminal CARD that anchors a CARD

domain, a central NACHT nucleotide-binding domain, LRR domain, a function-to-find domain (FIIND) and a C-terminal CARD. This structural layout is exclusive to NLRP1. NLRP1 can directly activate caspase-1 without recruiting ASC, although the bypass of ASC greatly declines the formation of the inflammasome complex and the processing of IL-1β (Faustin *et al.*, 2007, Proell *et al.*, 2013). It still remains unclear the method by which NLRP1 recruits ASC, as the mouse NLRP1 ortholog do not have PYD domains. It has been speculated that a CARD-CARD homotypic interaction between NLR and ASC recruits a first layer of ASC, resulting in the interaction with a second layer of ASC through interaction via PYR-PYR homotypic domain (Latz *et al.*, 2013). NLRP1 has been shown to sense the bacterial lethal toxin (LT) from *Bacillus anthracis* (Boyden and Dietrich, 2006). In addition, NLRP1 has been shown to directly bind to its ligand muramyl dipeptide, which is enough to activate the assembly of the inflammasome complex. However, NLRP1 seems to need the interaction of NOD2, another receptor for muramyl dipeptide, suggesting that NOD2 is an important component on the assembly of the NLRP1 inflammasome (Hsu *et al.*, 2008).

1.3.2.1.2. NLRP3 inflammasome

The NLRP3 inflammasome is the best characterised inflammasome. Unlike NLRP1, the NLRP3 has a typical tripartite structure organisation: an N-terminal pyrin domain, a central NACHT domain and a C-terminal LRR domain. The NACHT domain has ATPase activity and upon stimulation causes the receptor to oligomerise and recruit ASC via its pyrin domain. NLRP3 lacks a CARD domain therefore it can only recruit pro-caspase-1 via the adaptor molecule ASC (Davis *et al.*, 2011).

There are a plethora of triggers that activate NLRP3 including crystalline material, peptide aggregates, viral and bacterial PAMPs as well as damage-associated molecular patterns (DAMPs). NLRP3 has been shown to be activated by whole pathogens such as the fungi *Candida albicans* and *Saccharomyces cerevisiae*, the

Sendai virus, adenovirus and influenza virus (Schroder and Tschopp, 2010). NLRP3 has also been found to be activated by host-derived molecules such as extracellular ATP that are sensed by the P2X7 receptor due to cell death (Mariathasan *et al.*, 2006). The NLRP3 inflammasome also activates in response to fibrillar amyloid-β undergoing phagocytosis by microgilia. The secretion of the proinflammatory cytokine IL-1β in response to fibrillar amyloid-β, which has a key role in the pathogenesis of Alzheimer's disease, may play an integral role in the development of this disease (Halle *et al.*, 2008). With regards to the activation of NLRP3 inflammasome as a response to bacterial PAMPs, Kayagaki and co-workers (2011) showed that the activation of caspase-1 and IL-1β production, after the recognition of Gram-negative bacteria, relies on the inflammatory pro-caspase-11 in mice (human orthologues pro-caspase 4), which in turn results in a non-canonical NLRP3 inflammasome activation. This non-canonical activation is due to the requirement of three signals, instead of the standard two found in the IL-1β generation (see section 1.3.2.1), in response to Gram-negative bacteria. The first signal, which is found in the conventional model, is induced by the activation of NF-κB, which is promoted by TLR recognition of bacteria, which results on an up-regulation of NLRP3 and pro-IL-1β. The second signal involves two parts: the first is that the bacterial mRNA from live bacteria activates NLRP3; and the second is that the MyD88-independent pathway mediates the secretion of type-I IFNs, which in turn induce pro-caspase-11 expression and activation (Figure 1.13) (Kayagaki *et al.*, 2011, Latz *et al.*, 2013).

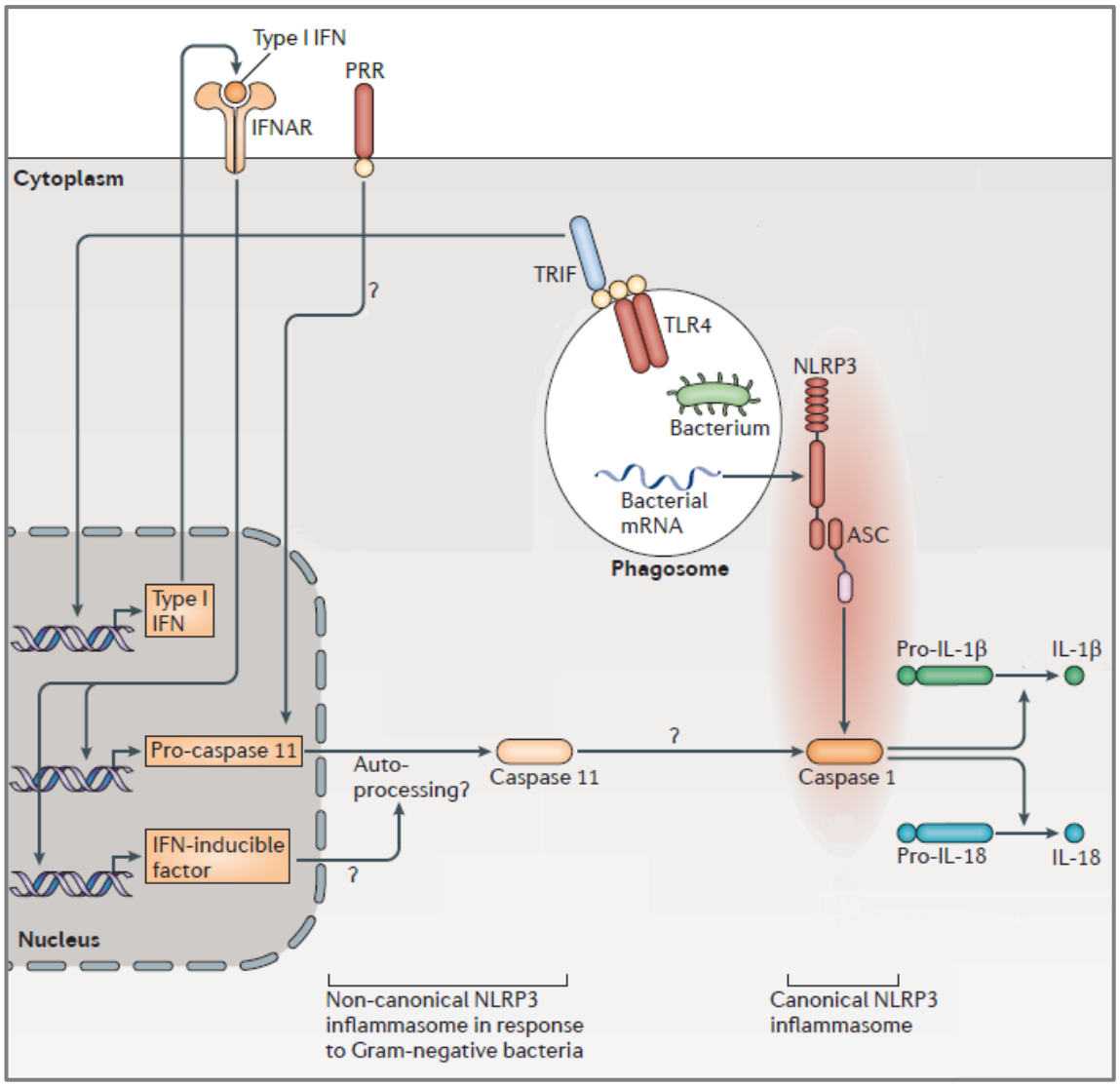


Figure 1.13. Canonical and non-canonical activation of IL-1β. NLRP3 needs supplementary co-factors for the induction of the bioactive form of IL-1β in response to Gram-negative bacteria. To successfully induce the production of IL-1β it is required a three step checkpoint. The first being the activation of TLR4 through TRIF which induces the secretion of type-I IFNs. The second is the activation of NLRP3 via live bacterial mRNA. The third step is via the MyD88-independent pathway which mediates the secretion of type-I IFNs, which in turn induce pro-caspase-11 expression and activation. Question marks show speculative pathways. Adapted from Latz *et al.* (2013).

1.3.2.1.3. NLRP12

There are some inflammasome sensors whose ability to form inflammasomes is as of yet undetermined. Although this is the case for NLRP12 (also known as Monarch-1 or PYPAF7), other functions have been described for this inflammasome sensor since its discovery in 2002 (Wang *et al.*, 2002). NLRP12 has been shown to be a positive regulator of dendritic cells and neutrophils migration, where NLRP12 knockout mice showed a significant reduction in the capacity of both these cell types to migrate to draining lymph nodes (Arthur *et al.*, 2010). NLRP12 has also been shown to be a negative regulator of non-canonical NF-κB signalling. The canonical pathway, as mentioned before, is activated through the IκB-kinase (IKK) complex which is made up of NEMO, IKK-α and IKK-β. IκB is then phosphorylated and undergoes proteosomal degradation, causing the release of NF-κB which induces the production of proinflammatory cytokines. On the other hand, the non-canonical pathway is dependent on the NF-κB-inducing kinase (NIK) which activates IKKα, and in turn leads to a nuclear accumulation of p52-containing NF-κB that induces the production of proinflammatory cytokines. Lich *et al.* (2007), showed that in *in vitro* analysis of monocytes that NLRP12 is a negative regulator of NF-κB. It was suggested that this NLR protein has the ability to associate with NIK, which suppresses non-canonical NF-κB activation (Lich *et al.*, 2007). In 2012, experiments with NLRP12-deficient mice demonstrated that these were very susceptible to inflammation. The authors concluded that NLRP12 functions as a NF-κB negative regulator through its interaction with NIK and TRAF3. This shows that this inflammasome sensor is important in regulating inflammatory responses (Allen *et al.*, 2012).

1.3.2.2. NOD1 and NOD2

Nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) and NOD2 play a major role in regulating NF-κB and MAP kinases, NOD1 being one of the very

first NLRs to be described (Inohara *et al.*, 1999). NOD1 and NOD2 recognise fragments of peptidoglycan, a major component of bacterial cell walls. NOD1 recognises γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard *et al.*, 2003), whereas NOD2 detects, like NLRP1, muramyl dipeptide (MDP) (Girardin *et al.*, 2003). NOD1 is thought to be responsible for the detection of Gram-negative bacteria, as iE-DAP is only found in Gram-negative peptidoglycan. On the contrary, NOD2 is a more general detector, as MDP is found in both Gram-negative and Gram-positive bacteria. Both these proteins also recognise various other pathogens from *Escherichia coli, Chlamydia pneumonia* and *Pseudomas aeruginosa*, detected by NOD1, to *Streptococcus pneumonia, Mycobacterium tuberculosis* and *Salmonella typhimurium*, by NOD2 (Kufer *et al.*, 2006, Kumar *et al.*, 2011).

The structure of NOD1 and NOD2 consists of a C-terminal LRR, a central nucleotide binding oligomerization domain, and an N-terminal domain containing either one (NOD1) or two (NOD2) CARDs. Upon ligand binding, NOD1 and NOD2 initiate oligomerization which leads to the recruitment of the adaptor molecule RIP2 via their respective CARD domains by homophilic interactions. NEMO is recruited to RIP2, which causes the phosphorylation of IKKβ releasing NF-κB to induce transcription of inflammatory cytokines (Figure 1.15). RIP2 can also recruit TRAF3 which leads to the activation of TBK1 and IKKε. This causes IRF3 and IRF7 to form hetero- or homodimers, translocate to the nucleus in order to induce type-I IFN expression (Kersse *et al.*, 2011).

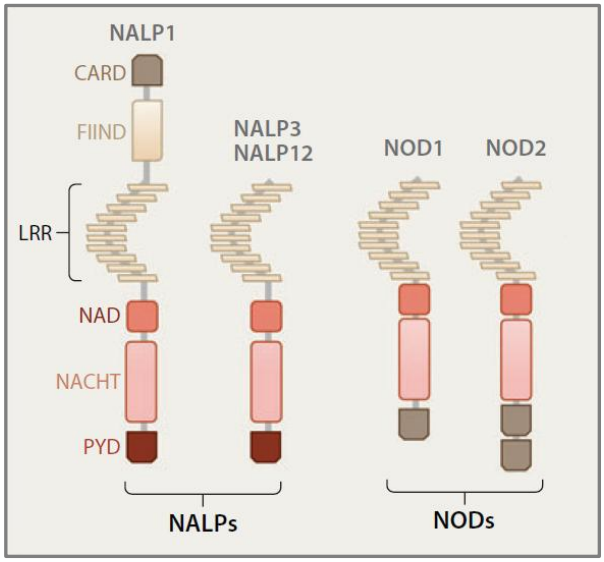


Figure 1.14. Structure of NOD-like receptors (NLRs). NLRs have three distinct domains: C-terminal leucine-rich repeats (LRRs), a central oligomerization domain (NACHT) and an N-terminal CARD or/and PYD domain. Most NLRs also contain a NACHT-associated domain (NAD) C-terminal of the NACHT domain. Adapted from Martinon *et al.* (2009).

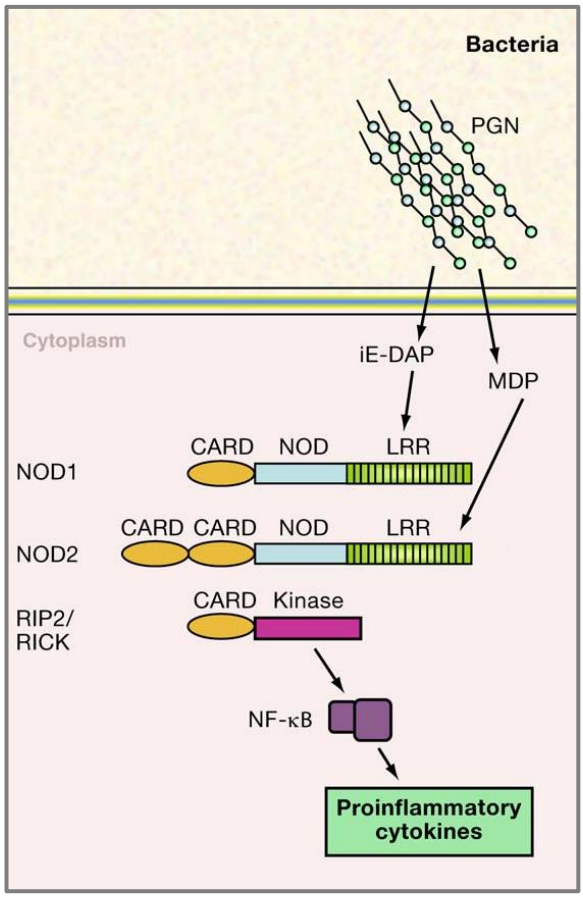


Figure 1.15. NOD1 and NOD2 proteins detect peptidoglycan (PGN) from Gram-negative (NOD1) and both Gram-negative and –positive (NOD2) bacteria. NOD1 and NOD2 recognise iE-DAP and MDP, respectively, and through the adaptor molecule RIP2 activate NF-κB which induces the transcription of proinflammatory cytokines. Adapted from Akira *et al.* (2006).

1.4. The innate immune response in sepsis

Sepsis is a clinical disorder that is an often-fatal systemic inflammatory response to a microbial infection. This medical condition is paradoxical, seeing as the mechanism that has been designed to protect the host against bacterial infection is the main source of injury and damage to the host. Bacterial products, such as lipopolysaccharide (LPS) form Gram-negative bacteria, which are recognised by the host leading to an oversecretion of cytokines that seem to be damaging to the host.

The pathophysiology of sepsis seems be a product of a multi-system disorder, where septic patients seem to have an uncontrolled initiation of coagulation, fibrinolysis and inflammation (Figure 1.16). If these cascades are not reversed, the development of sepsis is expected, resulting in multi-organ failure and likelihood of death.

The onset of sepsis triggers the hosts inflammatory response which in turn induces the production of inflammatory mediators, such as TNF-α and IL-1β, which have detrimental effects on the vascular endothelium. These pro-inflammatory cytokines induce the expression of tissue factor (TF), which promotes the coagulation cascade. TF interacts with factor VIIa, forming the complex VIIa-TF that activates factors X and IX. Ultimately the coagulation cascade leads to the formation of fibrin aggregates and, with incorporated platelets, form stable clots that play a major part on the initiation of sepsis. Multiple organ failure is thought to be directly related to this coagulation cascade, which manifests as disseminated intravascular coagulation (DIC), and microvascular thrombosis (Levi and Ten Cate, 1999).

Another system that becomes uncontrolled due to the onset of sepsis is the fibrinolysis cascade. This system is used to remove clots, and unlike the coagulation cascade, which becomes overexpressed during sepsis, fibrinolysis becomes suppressed. In health, plasmin is the main effector molecule of fibrinolysis. Plasmin production is induced when tissue plasminogen activation factor (t-PA) triggers the conversion of

plasminogen to plasmin, which in turn is able to degrade fibrin and the clots. The fibrinolysis cascade is tightly controlled by two inhibitors: the plasminogen activator inhibitor (PAI)-1, which is produced by endothelial cells and platelets in order to inhibit t-PA; and the thrombin-activatable fibrinolysis inhibitor (TAFI). Septic patients have this system compromised, where as a result of bacterial components, the levels of PAI-1 and TAFI are increased. The suppression of this system also decreases the levels of protein C, which is an inhibitor of PAI-1 and t-PA. (Bajzar *et al.*, 1996, Hotchkiss and Karl, 2003).

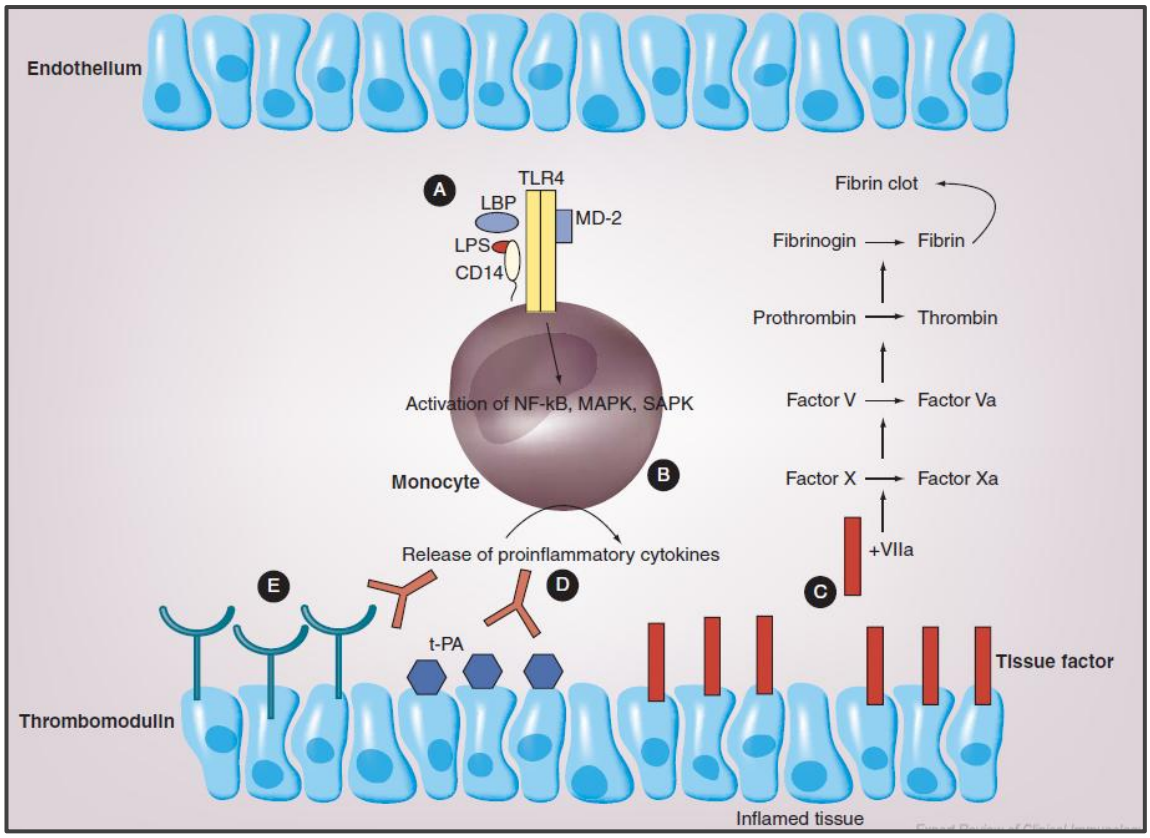


Figure 1.16. Uncontrolled cascades of inflammation, coagulation and fibrinolysis. A) LBP-LPS complexes bind to CD14 and MD2 and are transferred to the TLR4 homodimer; B) Production of pro-inflammatory cytokines; C) Proinflammatory cytokines lead to the initiation of the coagulation cascade; D) Proinflammatory cytokines also inhibit the function of t-PA, a key effector of fibrinolysis as well as E) thrombomodulin which activates protein C pathway. The combination of the uncontrolled cascades results in loss of homeostasis and DIC. Adapted from Triantafilou and Triantafilou (2006).

The combination from the uncontrolled cascades of inflammation, coagulation and fibriolysis, seen in patients with sepsis, result in a loss of homeostasis. As the body attempts to regain internal equilibrium, most endogenous modulators that are acting towards the normalisation of the body are consumed and depleted. This results in continuous endothelium injury resulting in a broad organ failure and eventually leading to death. Thus, the search for new therapeutic interventions for sepsis continues and the innate immunity field has been a key target for this purpose.

1.4.1. Therapeutics

Patients with severe sepsis and septic shock manifest striking heterogeneity including age, underlying diseases, diverse microbial etiology, local infection site and genetic makeup. This problem remains a major hurdle which makes the discovery of new therapies for severe sepsis and septic shock especially difficult. Over the past 4 decades, the situation for septic patients has improved significantly, and yet this improvement is almost solely due to advances in the supportive care given to critically ill patients and the use of antimicrobial agents (Suffredini and Munford, 2011).

One of first clinical trials of the sepsis hypothesis was published in 1976 by William Schumer. In this study 172 patients with septic shock were either treated with steroid or saline: 43 received dexamethasone (DMP), 43 received methylprednisolone (MPS), and 86 received saline. William Schumer reported that administration of a high dose of steroids could reduce mortality from 38.4% to 10.4% (Schumer, 1976). Forty years later, the use of high doses of steroids has been mostly abandoned, whilst the efficacy of lower doses still remains uncertain (Marshall, 2014, Minneci *et al.*, 2009, Suffredini and Munford, 2011).

Hundreds of millions of dollars have been spent in more than 100 Phase II and Phase III clinical trials, with over 30,000 patients, to test and develop new immunomodulating agents, anti-inflammatory agents and anti-endotoxin agents (Marshall, 2014, Opal *et*

al., 2014). The result of this effort and expenditure, with one momentary exception, has lead to no new treatments or drugs coming to the market.

The molecular pathophysiology of sepsis is very diverse. Consequently, the targets for therapeutic interventions have had numerous approaches. Strategies have included selective neutralisation of bacterial products such as endotoxin; non-selective suppression of inflammation with the use of corticosteroids or non-steroidal anti-inflammatory drugs (NSADs) such as Ibuprofen; the inhibition of host inflammatory mediators such as tumor necrosis factor (TNF), interlukin-1 (IL-1), platelet-activating factor (PAF), and nitric oxide (NO); the administration of proteins such as granulocyte colony-stimulating factor (G-CSF) and interferon γ; and the administration of anticoagulant molecules such as activated protein C (APC), anti factor pathway inhibitor (TFPI), antithrombin, thrombomodulin, or heparin (Marshall, 2014).

1.4.1.1. Anti-endotoxin

In the 1970s and 1980s attention focused on neutralizing Gram-negative bacterial endotoxin or lipopolysaccharide (LPS), and anti-endotoxin antibodies were tested in several trials. Promisingly, the first human trial of anti-endotoxin showed initial positive results, where Ziegler *et al.* (1982) used J5 antiserum containing polyclonal antibodies directed against the core glycolipid structure of LPS, but such results were never reproduced. Clinicians also tested 2 anti-LPS monoclonal antibodies – HA-1A, a human antibody; and E5, a murine antibody. HA-1A is a human monoclonal IgM antibody that binds specifically to the lipid A domain of LPS. Ziegler *et al.* (1991) showed improved mortality with the use of HA-1A therapy in 200 patients with proven Gram-negative sepsis. On the contrary, the 343 septic patients without culture proven Gram-negative bacteria showed no treatment benefit. With regards to E5, another monoclonal IgM anti-endotoxin antibody, showed improved mortality and the potential to reverse organ failure (Greenman *et al.*, 1991). In a follow-up study, Bone *et al.*

(1995) showed that patients with suspected or proven Gram-negative sepsis treated with E5 had no difference in mortality. Further larger clinical trials of both HA-1A (McCloskey *et al.*, 1994) and E5 (Angus *et al.*, 2000) have failed to show any benefits.

A novel approach to the treatment of sepsis involved the enterobacterial common antigen (ECA), a specific glycophospholipid surface antigen shared by all members of the family *Enterobacteriaceae*. Organisms from this family are responsible for about 70% of proven Gram-negative infections. ECA has a close association with LPS in the bacterial cell wall. A human monoclonal IgM antibody, MAB-T88, directed at the enterobacterial common antigen was selected for its affinity and neutralisation of ECA. Albertson *et al.* (2003) evaluated the efficacy of MAB-T88 in 826 patients with sepsis in a prospective, randomized, double-blinded, placebo controlled, multicenter. There was no difference in mortality in all the patients enrolled (MAB-T88 37% vs. placebo 34%).

A more specific endotoxin neutralizing agent, recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$), seemed to prevent morbidity from meningococcal sepsis when tested in children. $rBPI_{21}$ is a recombinant 21-kDa modified fragment of human BPI, a natural protein stored in the neutrophil granules that binds and neutralises the effects of LPS. Although $rBPI_{21}$ appeared to prevent morbidity, it did not improve survival rate (Levin *et al.*, 2000).

In recent times, 3 novel anti-endotoxin compounds were tested in clinical trials but unfortunately all failed to meet their primary goals.

In 2009, Dellinger and co-workers assessed the efficacy of a phospholipid emulsion (GR270773) in Gram-negative severe sepsis. This phospholipid emulsion, which binds and neutralizes endotoxin, was administered to 1379 patients with confirmed or suspected Gram-negative sever sepsis. Regrettably, this multicenter clinical trial stopped early because of adverse and life threatening events. Treatment with

phospholipid emulsion did not reduce mortality, nor did the onset of new multi organ failure (Dellinger *et al.*, 2009).

The quest to find a new therapeutic approach led clinicians to attempt to inhibit toll-like receptor 4 (TLR4), which is expressed on the surface on immune cells, and binds to LPS resulting it to initiate the release of proinflammatory cytokines. The first attempt was made with TAK-242, a small-molecule which functions as an inhibitor of the TLR4 signalling pathway after TLR4 binds with LPS. Promising preliminary results, on a septic animal model, showed a decrease in cytokine levels. Rice *et al.* (2010) evaluated the efficacy of TAK-242 in a randomized, placebo-controlled trial of patients with severe sepsis shock or respiratory failure. In this trial TAK-242 failed to suppress cytokine levels, in particular IL-6, and showed no difference in 28-day mortality compared to placebo in affected patients. The second and most recent try was with eritoran tetrasodium (E5564), a synthetic lipid A antagonist that blocks LPS from binding at the cell surface MD2-TLR4 receptor. Phase 1 trial showed that eritoran blocked the effects of LPS and cytokine (TNF-α and IL-6) responses, and clinical illness in healthy volunteers (Lynn *et al.*, 2003). In a phase 2 trial of E5564 treatment, in patients with high risk of death, positive results showed a lower mortality rate against placebo (Tidswell *et al.*, 2010). Opal *et al.* (2013) performed a multinational phase 3 trial in 197 ICUs in order to determine if eritoran would reduce sepsis-induce mortality. The result was negative compared with placebo.

In recent years polymyxin B (PMX) has been a target for possible anti-endotoxin therapy. Polymyxin B is a polypeptide antibiotic that has the ability to bind and neutralise endotoxin. Intravenous injection of PMX has significant nephrotoxic and neurotoxic effects. However, a novel therapeutic strategy where PMX has been successfully adsorbed to polystyrene-derivative fibers (PMX-F) in a hemoperfusion medical device. Immobilized PMX does not leak out into the blood stream, removing its known toxic effects (Klein *et al.*, 2014). Cruz *et al.* (2007) have published a systematic

review of 28 trials using PMX direct hemoperfusion (PMX-DHP) to treat patients with severe sepsis and septic shock. Although the authors commented that the analysed studies were of “suboptimal quality”, i.e. heterogeneity amongst the trials, the results appear to have a favourable outcome on mean arterial pressure, dopamine use, oxygenation (PaO_2/FiO_2) ratio and mortality. In 2009, the Early Use of Polymyxin B Hemoperfusion in Abdominal Sepsis (EUPHAS), a randomized trial of 64 patients in 10 Italian ICUs, showed significant improvements in hemodynamics and organ dysfunction. It also reduced mortality from 53% in standard therapy to 32% in the PMX-DHP group (Cruz *et al.*, 2009). Although positive, these results might be skewed as the trial was stopped early after an interim analysis showed a mortality difference in the PMX-DHP group. Also, the trial was not blinded and patients were selected based on evidence of intra-abdominal sepsis where endotoxin levels were not measured prior enrolment for therapy. In light of these limitations, the EUPHAS investigators have called for further studies into the effectiveness of PMX-DHP (Kellum and Uchino, 2009). The ongoing trial, Evaluating the Use of Polymyxin B Hemoperfusion in a Randomized controlled trial of Adults Treated for Endotoxemia and Septic shock (EUPHRATES), is the latest trial investigating the safety and efficacy of PMX-DHP based on mortality at 28-days in subjects with septic shock who have high levels of endotoxin and are treated with standard medical care plus use of the PMX medical device (Toraymyxin PMX cartridge), versus subjects who receive standard medical care alone. This trial began in June 2010 and is expected to be completed by July 2016. EUPHRATES is a multi-centered, placebo-controlled and blinded trial which is comprised of 360 patients from 50 ICUs in the United States and Canada. In the first quarter of 2014, the second interim analysis recommended to continue the trial (Klein *et al.*, 2014).

1.4.1.2. Corticosteroids

From the hypothesis that septic shock and organ failure developed from the host inflammation, clinical trials turned their efforts to anti-inflammatory interventions that would improve outcomes. Glucocorticoids have been studied in 21 trials over 4 decades with the objective to reduce inflammation in patients with sepsis. From 1963-1988 a meta-analysis of 9 trials showed that short doses of high-dose of steroids (median dose 23,975 mg of hydrocortisone equivalents over a median of 1 day) increased mortality and worsened secondary infections. In contrast, 11 small trials conducted after 1997, which involved longer courses of lower-dose steroids (median dose, 1,209 mg equivalents of hydrocortisone over a median of 6 days) were associated with beneficial treatment effects, including improved shock reversal and survival (Minneci *et al.*, 2004, Minneci *et al.*, 2009). One of the main limitations of studies of low doses of steroids in septic shock is that the beneficial effects were found mainly in small trials. Furthermore, the Corticosteroid Therapy of Septic Shock (CORTICUS), the largest and most recent randomized controlled trial, was studied in a low-risk population (Minneci *et al.*, 2009, Sprung *et al.*, 2008). This multicenter trial reported that low doses of hydrocortisone did not improve survival or reversal of shock in patients with septic shock (Sprung *et al.*, 2008). The beneficial effects of low-dose steroids needs to be confirmed in a large, multicenter trial of high-risk patients. Until this data is available, the decision to administer low-dose steroids for septic patients should be based on individual patients severity of illness and potential risk from steroid therapy (Marik *et al.*, 2008, Minneci *et al.*, 2009).

1.4.1.3. Anti-inflammatory therapies

Following the discovery of tumor necrosis factor (TNF-α) and interleukin 1 (IL-1β) it was believed that these pro-inflammatory cytokines are responsible for the inflammatory response that leads to hypotension, activation of the clotting cascade and organ

dysfunction, characteristics found in septic patients. Non-selective high-dose steroid therapy was abandoned in favour of testing agents that could selectively dampen these proinflammatory mediators.

1.4.1.3.1. Anti-TNF-α therapy

Due to its pivotal position in the inflammatory response to endotoxin-induced shock, controlling the levels of circulating TNF-α makes an attractive therapeutic target. Investigations of this therapeutic approach in patients were initiated in the 1990s, during which numerous strategies were developed in order to attenuate or neutralize excess circulating TNF-α. One of these approaches attempted to use TNF-α receptor constructs as soluble inhibitors of TNF-α bioactivity. There are two cell membrane-associated TNF-α receptors, p55 and p75, when cleaved from the cell membrane and released into circulation bind free TNF-α, preventing it from binding to target cells, consequently, not activating them. A p55 TNF-α receptor fusion protein (lenercept) was created by fusing the extracellular domain of the receptor to the Fc region of an immunoglobulin. Although there were encouraging results in preclinical and Phase II trials, in a larger multicenter Phase III trial, lenercept showed no significant effect on mortality in patients with severe or early septic shock (Abraham *et al.*, 2001a). Administration of a recombinant p75 fusion protein was also investigated. Although preliminary results in animal models, where it prevented death due to bacteraemia, when administered to patients with sepsis an unexpected increase in mortality was observed for treated patients (Fisher *et al.*, 1996).

Another strategy clinicians used in order to neutralize circulating TNF-α was the use of specific anti-TNF-α antibodies. Reinhart *et al.* (2001) investigated whether or not treatment with an anti-TNF-α monoclonal antibody, afelimomab, would improve survival in septic patients with IL-6 concentrations of >1000 pg/mL. This hypothesis was tested in a prospective, randomized placebo controlled trial, the RAMSES study. This study

had 446 participants (prospectively stratified from 944 patients: IL-6 levels >1000 pg/mL - 446 patients and <1000 pg/mL – 498) with high levels of IL-6. Patients treated with afelimomab showed a 3.7% reduction in mortality rate but this result did not reach statistical significance. A later study, the MONARCS trial, tested the same monoclonal antibody fragment in 2,643 patients with severe sepsis and elevated IL-6 levels. Out of the total number of patients, 998 had elevated IL-6 levels (>1000 pg/mL). The results from the MONARCS trial, a phase III trial, showed that the administration of afelimomab lowered circulating levels of TNF-α and IL-6, and accelerated the resolution of organ dysfunction. This study also found a reduction in mortality of 5.8% in patients with elevated IL-6 levels (IL-6 levels >1000 pg/mL: afelimomab 42.8% vs. placebo 48.6%. IL-6 <1000 pg/mL: afelimomab 26.4% vs. placebo 27.7%), demonstrating that afelimomab had a greater effect in patients at high risk of mortality (Panacek *et al.*, 2004). In a similar investigation, CytoFab, a polyclonal anti-TNF-α fragment antigen binding (Fab) IgG fragments, was evaluated for safety and effectiveness in 81 septic patients with either shock or two organ dysfunctions. Although CytoFab was effective in reducing levels of circulating TNF-α and IL-6, and in increase increasing ventilator-free days, it did not show a difference in mortality (Rice *et al.*, 2006). Recently, CytoFab was further developed into AZD9773 (also a polyclonal antibody to TNF-α), and in a phase IIa in 70 patients with severe sepsis, AZD9773 was well tolerated, had an acceptable safety profile, and higher dose treatment successfully reduced TNF-α concentrations to near-undetectable levels (Morris *et al.*, 2012). The evaluation of AZD9773 continued to a larger phase IIb study. This was a multicenter, randomized, double-blind, placebo controlled trial in 300 patients evaluating the efficacy of two intravenous dosing regiments of AZD9773. This phase IIb demonstrated that although AZD9773 decreased circulating TNF-α levels in patients with severe sepsis or septic shock, it had no statistically significant impact on ventilator-free days or mortality in comparison with the placebo group (Bernard *et al.*, 2014).

1.4.1.3.2. Anti-IL-1 therapy

IL-1 is a proinflammatory cytokine that is synthesized by monocytes, macrophages and neutrophils in response to TLR engagement and, with working with TNF-α, promotes the progression of the hyperimmune response typical of a septic patient. IL-1 gene transcription is normally accompanied by the transcription of the gene for IL-1 receptor antagonist (IL-1ra), which reversibly binds and competitively inhibits IL-1 receptors (Arend, 1991). This makes the modulation of IL-1ra, and subsequently IL-1, an attractive target for sepsis immunotherapy. Recombinant IL-1ra has been evaluated in three multicenter trials. Fisher *et al.* (1994b), in a phase II study, evaluated the use of human recombinant IL-1ra (rhIL-1ra) in the treatment of patients with sepsis. The results from this 99 patient trial showed that dose-depended administration of rhIL-1ra reduced mortality from 44% to 16% for treated patients. These encouraging results led to two subsequent phase III trials. The first, evaluated the use of rhIL-1ra in the treatment of 893 patients with sepsis, where there was not a statistical significant increase in survival for patients treated with rhIL-1ra compared with placebo (Fisher *et al.*, 1994a). Secondly, Opal and colleagues published a trial in 1997 where continuous infusion of rhIL-1ra to patients (n=696) with severe sepsis failed to demonstrate a reduction in mortality. This trial was terminated after an interim analysis revealed a low likelihood of showing a statistical difference in their primary endpoint, 28-day mortality (Opal *et al.*, 1997).

1.4.1.3.3. Platelet-activating factor therapy

The making of bioactive lipids from cell membrane components occurs alongside the transcription of pro-inflammatory cytokines. Phospholipase A_2 (PLA_2) catalyses the conversion of cell membrane phospholipids to arachidonic acid and lysophospholipds like platelet-activation factor (PAF). Receptors for PAF are expressed on platelets, neutrophils and monocytes and have been associated in priming, amplifying and

regulating the release of inflammatory mediators in sepsis (Dhainaut *et al.*, 1998). There have been two distinct strategies to neutralize PAF: PAF receptor antagonist BN52021 (Ginkgolide B); and inactivating PAF activity through PAF acetylhydrolase. In 1994, Dhainaut and co-workers evaluated the safety and efficacy of BN52021, a natural platelet-activating factor receptor antagonist (PAFra), in the treatment of patients with sepsis. Although the initial results seemed to be positive, where the mortality rate was 42% for the PAFra group compared to 51% in the placebo, a phase III trial in 609 patients showed that a 4-day administration of BN52021 failed to demonstrate a statistically significant reduction in mortality of patients with severe sepsis (Dhainaut *et al.*, 1998, Dhainaut *et al.*, 1994). Two other PAF receptor antagonists, TCV-309 and lexipafant, were tested but neither showed any improvement in mortality rates (Marshall, 2003). The other strategy exploits the fact that PAF is inactivated through the enzymatic action of PAF acetylhydrolase (PAF-AH). This enzyme accounts for all the PAF-inhibitory activity found in human blood. Septic patients have low circulating levels of PAF-AH, and a recombinant human enzyme (rPAF-AH, Pafase) has been tested in a phase II trial in 127 patients with severe sepsis. The results from this trial indicated that rPAF-AH was well tolerated and the mortality rate was lower in patients treated with rPAF-AH compared with patients receiving placebo (Schuster *et al.*, 2003). A phase III trial in 1,425 (2522 patients planed but trial terminated early) patients was undertaken in order to find out if rPAF-AH would decrease 28-dy all-cause mortality in patients with severe sepsis. Although it was well tolerated and not antigenic, the trial was stopped early as the results from this large phase III trial showed that rPAF-AH does not decrease mortality in patients with severe sepsis (rPAF-AH 25% vs. placebo 24%) (Opal *et al.*, 2004).

1.4.1.4. Nitric oxide

Nitric oxide (NO) has been found to have a variety of effects in the innate response to infection and also plays an important role in the development of vasodilatation during septic shock. Nitric oxide is continuously generated from L-arginine by nitric oxide synthase (NOS) and is found, in the healthy state, at low concentrations. Endotoxin and cytokines are associated with the increased production of nitric oxide, which in turn is associated with hypotension, decreased responsiveness to vasoconstrictors, and development of multiple organ dysfunction (Lopez *et al.*, 2004). The different isoforms of NOS can be partially inhibited, reducing the overproduction of nitric oxide. This may be a beneficial intervention for septic patients. The administration of NOS inhibitors, such as a methylated arginine analog, L-N-monomethyl argenine (L-NMMA), or by N-omega-nitro-L-arginine methyl ester (L-NAME), to patients with septic shock have shown to restore hemodynamics, increased blood pressure and a decrease in the need for vasopressors (Marshall, 2003). The safety and efficacy of N^G-methyl-L-arginine hydrochloride (546C88), a NOS inhibitor, was assessed in a phase II clinical trial in 312 patients with septic shock. The results demonstrated that patients with septic shock in the 546C88 treatment group showed an elevation of blood pressure, increase in resolution of shock, decrease in the requirement of vasopressors and a reduction in plasma nitrate concentrations (Bakker *et al.*, 2004, Watson *et al.*, 2004). A randomized, double-blind, placebo-controlled phase III clinical study of 546C88 was performed in order to assess its safety and efficacy in reducing mortality in patients with septic shock. Unfortunately this trial was stopped early by a data safety monitoring board, due to excess mortality in the 546C88 group (546C88 59% vs. placebo 49%) (Lopez *et al.*, 2004). An alternative approach to inhibit nitric oxide is the inhibition of guanylate cyclise with methylene blue. The administration of methylene blue to patients has shown little or no effect on cardiac output when administered in septic patients (Weingartner *et al.*, 1999).

1.4.1.5. Anticoagulant Therapy

The onset of severe sepsis not only results in acute activation of the inflammatory system, but also simultaneously stimulates the coagulation system and inhibits fibrinolysis. From an adaptive point of view, activating coagulation and inhibiting fibrinolysis can be seen as an effort to isolate the infection with the intention of reducing its spread throughout the body. However, in the process of severe sepsis, this results in a counterproductive action as the infection is likely to be spread throughout the bloodstream and the activation of the coagulation system results in diffuse microvascular thrombi with widespread endothelial damage and organ dysfunction. In health, the activation of coagulation is tightly regulated through the activity of three endogenous anticoagulant pathways: the tissue-factor-pathway-inhibitor (TFPI) pathway, the antithrombin pathway, and the protein C pathway. Sepsis causes the deregulation of each of these pathways – making them prime targets in the treatment of sepsis (Bernard and Bernard, 2012, Marshall, 2003).

1.4.1.5.1. Tissue factor and antithrombin

Tissue factor (TF) is a transmembrane cell surface receptor found in endothelial cells which acts to initiate the coagulation pathway. TF inhibitor (TFPI) is an endogenous serine protease inhibitor that has been tested in septic patients in order to protect microvasculature endothelium from coagulation. Tifacogin (recombinant TFPI (rTFPI)) has been tested in three phase II studies. The most recent one, tifacogin was tested in 210 patients and results showed that there was a reduction in circulating IL-6 levels and a trend toward reduction in 28-day all-cause mortality in the tifacogin group compared with placebo (Abraham *et al.*, 2001b). Tifacogin was tested in the phase III trial, OPTIMIST (optimized phase III tifacogin in multicenter international sepsis trial), evaluating its efficacy in reducing mortality in patients with severe sepsis. This trial

failed to show any benefit in mortality, and it was associated with an increase in serious adverse events with bleeding in the tifacogin group (Abraham *et al.*, 2003).

Antithrombin III (AT III), an anticoagulant which is synthesized in the liver, was the subject of sepsis therapy due to the fact that AT III levels are reduced in septic patients. A meta-analysis of three trials, published in the 1990s, recruiting 122 patients showed promising results – a 22.9% reduction in 30-day all-cause mortality in patients treated with AT III (Eisele *et al.*, 1998). However, an international multicenter phase III trial that recruited 2,314 patients was unable to show a difference in overall 28-day mortality (AT III 38.9% vs. placebo 38.7%). Furthermore, like tifacogin, high-dose of AT III was associated with an increased risk of haemorrhage (Warren *et al.*, 2001).

1.4.1.5.2. Activated protein C

So far, the only drug approved for the treatment of severe sepsis was Xigris (drotecogin alfa, a recombinant human activated protein C (rhAPC)). It was investigated due to its anti-inflammatory, anti-coagulant and profibrinolytic properties. After one positive study in baboons (Taylor *et al.*, 1987), the efficacy and safety of rhAPC for severe sepsis was evaluated in a randomized, double-blind, placebo-controlled, multicenter phase II trial – the PROWESS study. This trial was stopped early for efficacy, 28-day mortality was lower in the rhAPC group. Treatment was associated with a 6.1% absolute reduction (24.7% rhAPC vs. 30.8% control) and 19.4% relative risk reduction, with a relation noted between severity of disease and survival advantage (Bernard *et al.*, 2001). The drug was approved by the US Food and Drug Administration (FDA) for use in the most severely ill septic patients with APACHE II scores greater than 25. APACHE II (Acute Physiology and Chronic Heath Evaluation II) is a severity-of-disease classification system (score from 0 to 71, where higher scores correspond to more severe disease and higher risk of death) and is applied within 24 hours of admission of a patient to an ICU. However, treatment with rhAPC

was reported to have no beneficial effects along with an increased incidence of serious bleeding complications in low-risk population. In addition, the patients in this trial who had an APACHE II score greater than 25, and treated with rhAPC, did not show any survival benefit observed in the first phase III trial (Abraham *et al.*, 2005). Equally, a paediatric trial published in 2007 that evaluated 477 septic children did not find any beneficial effects (Nadel *et al.*, 2007). In light of these contradictory results, authorities decided to request another randomized, multicenter, placebo-controlled study in order to re-assess the benefits noted in PROWESS. This lead to a subsequent trial, PROWESS-SHOCK. Regrettably, the results were not reproduced and the treatment was voluntarily removed from the market by the manufacturer (Ranieri *et al.*, 2012).

1.4.1.5.3. Thrombomodulin

Thrombomodulin (TM), a transmembrane protein on the endothelial cell surface involved in the regulation of the coagulation system, is currently being targeted as a new therapeutic of sepsis. Recombinant human soluble thrombomodulin (rhTM) binds to thrombin to inactivate coagulation, and the thrombin-rhTM complex acts upstream in the activated protein C pathway leading to the production of activated protein C. Disseminated intravascular coagulation (DIC), resulting from a widespread activation of coagulation pathways has been linked with mortality in patients with severe sepsis. A control study, performed by Yamakawa *et al.* (2011), of 20 patients with severe sepsis-induced DIC treated with rhTM compared to 45 controls showed improved 28-day morality and organ dysfunction in those treated with rhTM (rhTM group 25% vs. control 47%). In order to determine the safety and efficacy of rhTM (ART-123) a phase IIb, international, multicenter, double-blind, randomized, placebo-controlled trial was undertaken. ART-123, a soluble recombinant human thrombomodulin, acts by reducing the thrombin-mediated clotting and enhances the production of activated protein. In this trial of 750 patients, the results showed an improved 28-day mortality rate of 3.8% (ART-123 group 17.8% vs. placebo group 21.6%). Furthermore, ART-123 is a safe

intervention in critically ill patients with sepsis and suspected DIC, and the findings of this trial support a phase III study (Vincent *et al.*, 2013).

1.4.1.3.4. Heparin

The underlying principle behind anticoagulant treatments is that certain factors (TFPI, AT and APC) are found at a low levels in septic patients, therefore with the use of recombinant technology these levels are brought back to a normal. On the contrary, heparin, a naturally occurring proteoglycan in the coagulation system, does not replenish what sepsis patients have depleted, it binds to and activates antithrombin, which in turn reduces thrombin production and fibrin formation. The HETRASE study was performed in order to determine the effects of heparin as a treatment for sepsis. This randomized, double-blind, placebo-controlled, single centre clinical trial evaluated the length of stay and change in organ dysfunction of 319 patients with sepsis. The results did not demonstrate any beneficial effects, including 28-day mortality rate (Jaimes *et al.*, 2009).

1.4.1.6. Immunostimulants

Sepsis is normally viewed as an excessive activation if the inflammatory cascade in response to microbial pathogens. From time to time, patients present a delayed hypersensitivity, increased immune cell apoptosis, reduced lymphocyte blastogenesis and suppression of major-histocompatibility-complex class I/II molecules, resulting in a immunological exhaustion (Hotchkiss and Opal, 2010). One of the main strategies in attempting to reverse this state of immune suppression has been the use of granulocyte colony-stimulating factor. The growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) act on myeloid cell precursors and stimulate them to develop into mature neutrophils. In 2003, a phase III clinical trial investigated the effect of G-CSF (filgrastim) in patients with sepsis. A previous phase I/II trial demonstrated that filgrastim had the ability to

increase neutrophils count and improve the resolution of shock, but the results from the phase III clinical trial showed that there was no difference in mortality (filgrastim 29% vs. placebo 25.5%) (Root *et al.*, 2003). A later clinical trial evaluated the effect of early administration of G-CSF in patients with septic shock. The results from this study involving 166 septic patients demonstrated no clinical benefit (mortality rate G-CSF 27% vs. placebo 25%) (Stephens *et al.*, 2008). Compared to G-CSF, GM-CSF demonstrates broader effects and induces proliferation and differentiation of monocytes/macrophages, induces the release of pro inflammatory cytokines and induces antigen presentation (Schefold, 2011). A randomized phase II trial evaluated whether GM-CSF therapy would improve survival in patients with severe sepsis. This study showed no apparent difference in survival rate of the 19 patients at 30 days (GM-CSF 80% vs. placebo 75%). There was also no improvement in the discharge both from the hospital and ICU stay (Presneill *et al.*, 2002). In 2009, Meisel and colleagues tested whether GM-CSF would reverse monocyte deactivation, a trait of sepsis-induced immunosuppression. This was carried out by treating patients with GM-CSF, or placebo, and measuring the HLA-DR expression on monocytes, a biomarker of sepsis-associated immunosuppression. The results from this trial show that patients in the GM-CSF group had HLA-DR expression normalized in 19/19 treated patients vs. 3/19 control. The mortality rate also improved from 21% placebo vs. 16% in GM-CSF group, although it did not reach statistical significance (Meisel *et al.*, 2009). A meta-analysis made by Bo and co-workers (2011) investigated the effects of G-CSF and GM-CSF therapy in septic patients. The authors found no evidence that supports the use of G-CSF or GM-CSF in patients with sepsis although they suggest the accomplishment of large, prospective, multicenter clinical trials in order to investigate the potential of these immunosuppressants (Bo *et al.*, 2011).

1.4.1.7. Other therapies

1.4.1.7.1. Statins

There are a number of ways in which statins may have the ability to affect the immune response in sepsis – from their ability to increase the physiologic concentrations of nitric oxide; the decrease in the production of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6; and antithrombic properties, decreasing the effect of the sepsis-induced coagulation system (Janda *et al.*, 2010). A multicenter cohort study examined the effect of statin use and clinical outcomes in patients with sepsis. The results from this study showed no differences in severe sepsis risk between statin users and non-users for either prior statin use (30.8% vs. 30.7%) or continued use (30.2% vs. 30.8%). There was also no mortality benefit for prior or continued statin use (Yende *et al.*, 2011).

1.4.1.7.2. Lactoferrin

Lactoferrin, a glycoprotein belonging to the transferrin family, is best known for its bactericidal activities within specific granules of human neutrophils. It is also secreted as a protein which plays an important role in the innate mucosal defence, acting as an antimicrobial agent along the epithelial surfaces. Talactoferrin alfa (TLF), a recombinant form of human lactoferrin, has similar properties and acts as a mucosal barrier maintaining the gastrointestinal properties. Oral TLF has been shown, in both animal and preclinical studies, to protect against gut damage and to reduce translocation of bacteria across the gut mucosa (Guntupalli *et al.*, 2013). A phase II randomized, double-blind, placebo-controlled study was carried out to evaluate the efficacy of TLF in patients with severe sepsis. The results from this study, which enrolled 190 patients (97 patients received TLF and 93 placebo), showed that the administration of TLF reduced 28-day all-cause mortality (TLF group 14.4% vs. placebo 26.9%). Unfortunately, a follow-up phase II/III clinical trial with oral TLF in adult sepsis

was stopped early when the placebo group had a better 28-day mortality than the TLF group (Guntupalli *et al.*, 2013, McCulloh and Opal, 2013).

1.4.2. Dysregulated innate immune responses in sepsis

A tight regulation of the innate immune system is key to keep the body alive. The loss of regulation or overstimulation of innate immunity can lead to detrimental and often-fatal symptoms. Septic shock is a prime example of a condition that reflects this overreaction of the hosts innate immune system, where bacterial components cause the over secretion of cytokines, which trigger a chain of damaging cascades that cause this condition. In septic patients, as a result of the uncontrolled cascades of inflammation, coagulation and fibrinolysis, the homeostasis of the host is compromised, which leads to vital organ failure and vascular collapse.

It is imperative to identify molecules that are able to modulate the innate immune responses, in order to reverse these often-fatal outcomes. A variety of virulence factors derived from bacteria has been shown to be able to modulate innate immune responses. The focus of this thesis is to characterise one such virulence factor from *Yersinia pestis.*

1.5. *Yersinia* species

The genus *Yersinia* is a member of the class *Gammaproteobacteria*, within the family *Enterobacteriaceae*, a heterogeneous group of Gram-negative aerobic bacilli (Hurst *et al.*, 2011). This genus has undergone extensive diversification through its evolution, and has 11 currently recognized species, three of which are human pathogens: *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. These three pathogenic *Yersinia* species differ from each other in their pathogenesis. Whilst *Y. pestis* is the aetiological agent for plague, *Y. pseudotuberculosis* and *Y. enterocolitica* are important causes of human diarrhoeal diseases (Duan *et al.*, 2014, Perry and Fetherston, 1997).

Y. pseudotuberculosis is primarily an animal pathogen (domestic and farm animals and birds) although it can occasionally cause mesenteric adenitis in humans. More rarely can cause sepsis. It is likely to be transmitted to humans via food contaminated with infected faeces. *Y. enterocolitica* causes diarrhoeal disease, terminal ileitis and mesenteric adenitis. Infection may be complicated by the onset of sepsis or reactive polyarthritis. Both *Y. pseudotuberculosis* and *Y. enterocolitica* are food-borne pathogens and are transmitted through the faecal-oral route from domestic animals, such as sheep, swine, rodents, dogs, cats, birds and wild animals (Duan *et al.*, 2014).

Y. pestis is a non-motile, Gram-negative, facultative intracellular coccobacillus that is primarily found in rodents and is transmitted to humans via infected fleas, specially the rat flea (*Xenopsylla cheopsis*). *Y. pestis* is very closely related to the gastrointestinal pathogen *Y. pseudotuberculosis*, and it has been proposed that *Y. pestis* evolved between 1,500 and 20,000 years ago from *Y. pseudotuberculosis* (Achtman *et al.*, 1999). It grows at temperatures between 4 and 40ºC and tolerates pH 5 to 9.6, although its optimal growth occurs at temperatures of 28ºC to 30ºC and pH 7.2 to 7.6 (Perry and Fetherston, 1997). *Y. pestis* is the cause of plague and is still endemic in some areas of the world. Unless quickly treated, bubonic plague can have a mortality rate between 50% and 90%. Diagnosis and appropriate treatment can reduce bubonic plague mortality to 5% to 15% (Prentice and Rahalison, 2007, Williamson, 2009).

1.5.1. Plague

Plague is an ancient disease and has been responsible for an estimate of 200 million deaths over several millennia (Perry and Fetherston, 1997). The causative agent is *Yersinia pestis* and it was first isolated by Alexandre Emile John Yersin (1863-1943) in 1894 during the Hong Kong epidemic. *Y. pestis* has been linked with three pandemics. The first began in Pelusium, Egypt, and it is known as the Justinian plague (A.D. 541 to 767). The second pandemic, famously known as the Black Death, occurred from 1336

to the early 19th century. It spread from the Caspian Sea to all of Europe, killing up to a third of the European population. The third pandemic *Y. pestis* pandemic began in the middle of the 19th century in the Chinese province of Yünnan and spread globally. Most of the millions of deaths in this pandemic were in India and China, and only in 1950 was it finally considered over (Achtman *et al.*, 1999, Perry and Fetherston, 1997).

More recently, two epidemics of plague have occurred in India, between September and October 1994, showing that plague is not eradicated and can still be a contemporary problematic disease (Perry and Fetherston, 1997). Plague is one of the World Health Organisation (WHO) quarantinable diseases, and at least 1000 to 5000 human cases of plague and 100 to 200 deaths are still reported to the WHO annually (Stenseth *et al.*, 2008, Titball *et al.*, 2003).

1.5.2. *Yersinia pestis* virulence factors

The success of *Yersinia pestis* in causing these pandemics lies with the fact that it possesses several plasmids that encode virulence factors that aid the survival and dissemination through its host. These include a 70 kb plasmid (pCD1), which is common to all three human pathogen *Yersinia* species, and two unique plasmids to *Y. pestis*, a larger 100 kb termed pMT1 and a smaller 9.6 kb plasmid termed pPCP1 (Hu *et al.*, 1998).

The pCD1 (calcium dependence) plasmid, also known as pYV, contains genes which encode for the type III secretion system (T3S). This system comprises of *Yersinia* outer proteins (Yops) and their chaparones, V antigen and other regulatory proteins required for the bacterium to inject toxic substances into mammalian cells and essential for virulence. In all three species of *Yersinia* there are an array of genes that make part of a multicomponent virulence regulon, known as the low-calcium response (Lcr), which is instrumental in regulating the expression of the virulence genes (Hu *et al.*, 1998, Perry and Fetherston, 1997).

The pMT1 (murine toxin), also known as pFra in some strains, carries genes for the production of two proteins, Fraction-1 antigen (F1-antigen) and a phospholipase D (PlD). The 15 kDa capsular protein Fraction-1 (F1) antigen, also known as Caf1, is a capsule-associated protein which has antiphagocytic properties. F1-antigen is under the influence of the *caf* operon which is usually expressed at 37ºC, which regulates the assembly and anchoring of the F1 onto the outer membrane, where forms a large homopolymer (>200kDa) in a stacked circular structure composed of heptamers (Tito *et al.*, 2001). Not only is F1-antigen thought to have an anti-phagocytic effect, but also it seems it has an important role in bacterial transmission as it aids the adhesion to epithelial cells (Liu *et al.*, 2006). Phospholipase D is important in colonization of the flea gut and it appears to be needed to protect the bacterium from antibacterial factors in the flea midgut (Perry and Fetherston, 1997, Prentice and Rahalison, 2007).

Finally the pPCP1 (pesticin, coagulase, plasminogen activator) plasmid encodes for a collection of proteins which aid the propagation of *Y. pestis* in the mammalian host. These proteins include pesticin, coagulase and plasminogen activator (PLA), which collectively appear to be important for the dissemination of *Y. pestis* by the breaking-down of the physical barriers, i.e. endothelial and cell membrane, in order to achieve a successful transmission. PLA, a surface protease, is an essential virulence factor that activates plasminogen to plasmin, which cleaves fibrin clots and extracellular matrices, resulting in a facilitated migration of *Y. pestis* into mammalian endothelial cells (Lahteenmaki *et al.*, 2001, Perry and Fetherston, 1997).

1.5.3. LcrV or Virulence (V) antigen

LcrV, also known as V antigen, is a 326-residue, 37 kDa soluble protein encoded by the *lcrV* gene in the pCD1/pYV plasmid, which is common in all three human pathogenic *Yersinia* species. V antigen was the first antigen to be associated with full virulence of *Y. pestis*, and not only plays vital role in regulating the translocation of

Yops, but also has anti-host functions of its own, making it a multifunctioning protein that is crucial for virulence, and is also required as an active and passive mediator of disease resistance (Fields *et al.*, 1999, Pettersson *et al.*, 1999). The first crystal structure of *Y. pestis* LcrV, at a 2.2 Å resolution, was determined by Derewenda *et al.* (2004). Recently, Chaudhury and colleagues (2013) refined the structure of *Y. pestis* LcrV and reported at 1.65 Å resolution (figure 1.17).

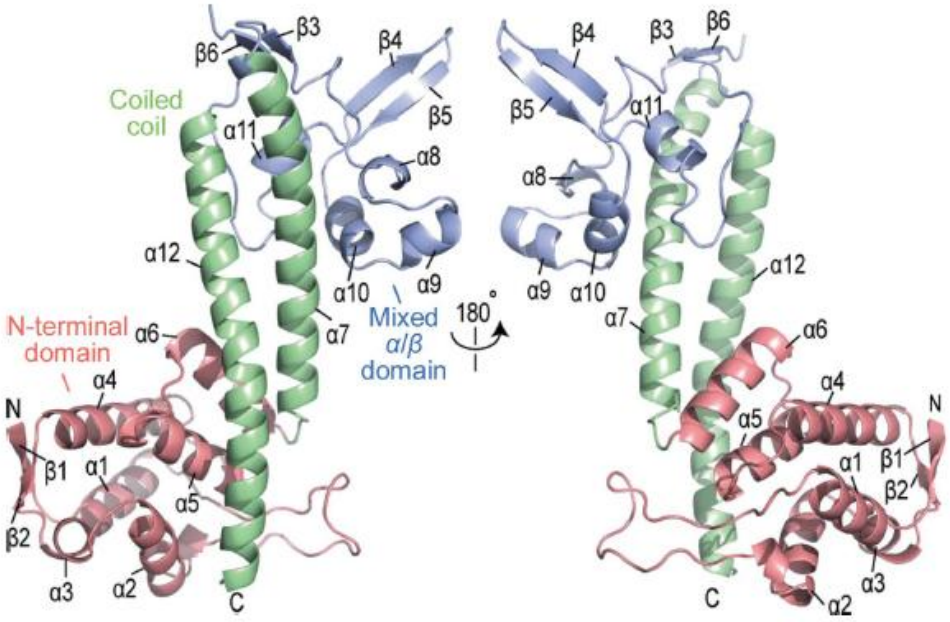


Figure 1.17: Structure of *Yersinia pestis* LcrV at 1.65 Å resolution. In pale pink the N-terminal globular domain, in green the central coiled-coil domain and in light blue the less structured mixed α/β domain. Adapted from Chaudhury *et al.* (2013).

Y. pestis LcrV was thought to be entirely intracellular, but it has been shown to be secreted into the extracellular medium in low-calcium environments, at 37ºC, regardless of cell contact. It most likely does so when it dissociates from the *Yersinia* secretion apparatus (Perry and Fetherston, 1997, Williamson and Oyston, 2013). LcrV also regulates the opening of type III secretion (T3S) channel called the injectisome, through which anti-host and cytotoxic factors can be translocated into the host cell. The

injectisome is composed of a basal body embedded in the two bacterial membranes and an external needle protruding from the bacterial surface (Fields *et al.*, 1999, Pettersson *et al.*, 1999). Broz and co-workers determined the molecular architecture of the *Y. enterocolitica* LcrV injectimsome tip. They showed that the tip complex is formed by three to five LcrV monomers, with a pentamer being the best fit for an atomic structure (Broz *et al.*, 2007).

1.5.3.1. Immnunomodulatory activity of V antigen

V antigen has previously shown immunosuppressive capabilities, thus making it a protein that is greatly researched. In 1995, Leary and colleagues showed that *Y. pestis* LcrV suppressed the production of the cytokines TNF-α and IFN-γ (Leary *et al.*, 1995). Schmidt *et al.* used a recombinant polyhistadine LcrV (rVagHis) derived from *Y. enterecolitica* O8 to show that LcrV suppressed TNF-α in Balb/c mice, not by direct effect on macrophages, but with the presence of activated T-cells. It does so, not with LcrV-induced IL-10 expression, nor by cell-to-cell contact between macrophages and T-cells, but with an LcrV-induced soluble factor produced by T-cells. Neutralising anti-IL-10 antibodies did not stop or diminish TNF-α suppression. The resulting suppression appears to occur due to either a decrease of transcription of TNF-α or increase of mRNA degradation (Schmidt *et al.*, 1999).

Studies carried out by Sing and colleagues have provided insights in how V antigen modulates innate immune responses. Their results appear to contradict the previous findings by showing that IL-10 is induced in response to *Y. enterocolitica* O8 recombinant LcrV (rLcrV) stimulation in both *in vitro* models of murine peritoneal macrophages (from C3H/HeJ mice) and human monocytic Mono-Mac-6 cells lines. Despite not using T-cells in these *in vitro* models it was shown that IL-10 is required for the successful TNF-α suppression. However, they did not disprove that an alternative mechanism of IL-10 independent TNF-α suppression through the rLcrV-dependent

secretion of an unidentified soluble factor from T-cells does not occur. They further suggested that TLR4 is not involved in this process by stimulating mice cells with a non-functional TLR4 (C3H/HeJ, Balb/c LPSd) and without TLR4 (C57BL/10sCr) with V antigen, which still resulted in TNF-α suppression (Sing *et al.*, 2002a). Sing *et al.* later that year demonstrated that *Y. enterocolitica* O8 rLcrV signals IL-10 production in a CD14-TLR2-dependent manner in both murine and human cells (CD14-TLR2-contrasfected HEK 293), showing that both TLR2 and CD14 are necessary for IL-10 induction and identifying a new ligand specificity of TLR2 (Sing *et al.*, 2002b).

Later on, Sharma and colleagues demonstrated that, in Balb/c mice peritoneal macrophages, LcrV innate immune recognition involves TLR2 as well as TLR6, but not TLR1 and that it results in the inhibition of transcription of some cytokines and chemokines (Sharma *et al.*, 2005, Sharma *et al.*, 2004, Sodhi *et al.*, 2005).

Reithmeier-Rost and co-workers (Sings group) investigated whether *Y. enterocolitica* O8 rLcrV had the same effects they had observed with wild-type C57BL/6 mice peritoneal macrophages in the human monocytic cell like Mono-Mac-6. They verified once again that TNF-α secretion was suppressed and IL-10 expression was induced in response to rLcrV. They expanded these findings by stimulating the human and murine cells by collecting and analysing samples during a 24 hour period, where they observed that rLcrV induced TNF-α secretion, which peaked at 3h and subsequently decreased until reaching baseline levels past 24h in the human cells. In addition, they found that rLcrV induced IL-10 secretion was detected after 6h and kept increasing, reaching peak after 24h. IL-10 secretion was also confirmed to be induced in a TLR2/CD14-dependent manner following rLcrV stimulation in human Mono-Mac-6 cells, thereby confirming that this dependence applied to more than one type of human cells. An additional finding included rLcrV (TLR2 agonist) inducing auto-, homo- and heterotolerance, and that rLcrV induces IL-10 secretion at long range, without requiring

cell-to-cell contact and that rLcrV does not influence TLR2 or TLR4 expression in Mono-Mac-6 cells (Reithmeier-Rost *et al.*, 2004).

Later on, the same group wanted to verify whether *Y. pestis* LcrV acted the same way as *Y. enterocolitica* O8 LcrV by inducing IL-10 production in a TLR2/CD14-dependent manner. They infected cells of certain types and species with either *Y. pestis* LcrV or *Y. enterocolitica* O8 LcrV, and found that compared to *Y. enterocolitica*, *Y. pestis* LcrV resulted in a dramatically weaker TLR2/CD14-mediated response, meaning there was a lower secretion of IL-10 and TNF-α. Other findings of theirs led them to conclude that the presence of TLR2 does not change the final outcome of *Y. pestis* infection of mice and that since *Y. pestis* LcrV/TLR2 interaction is relatively weak, that TLR2-dependent IL-10 induction by LcrV does not contribute to virulence of *Y. pestis* (Reithmeier-Rost *et al.*, 2007).

1.5.3.2. *Yersinia pestis* V antigen protective fragments

Previous studies have shown that, not only the entire, but also small regions of *Yersinia pestis* V antigen can provide protection against the plague. In 1997, Hill and colleagues tested a series of V antigen truncates in order to test their immunogenicity and functionality. They suggested that *Y. pestis* V antigen contains a number of protective epitopes that are located in a central region between amino acids (a.a.) 135 and 275 (Hill *et al.*, 1997).

Subsequently, Vernazza *et al.* (2009) aimed to identify the minimum protective fraction of *Y. pestis* V antigen that could provide protection against plague. In order to do so, they generated small regions of the V antigen, residing between the central a.a. 135-275 fragment, by removing unwanted parts of the structure and producing recombinant proteins. Based on analysis, models of a.a. 135-275 (V1), 168-275 (V2), 175-275 (V3), 135-268 (V4), 135-262 (V5) and 135-275 Δ218-234 (V6) (Table 1.4, Figure 1.18) were generated and probed for the preservation of a protective epitope using a protective

monoclonal antibody and the protection against *Y. pestis* in mice was evaluated (Vernazza *et al.*, 2009).

Fragment	Deletion type	Amino acids
V1	-	135-275
V2	N-terminal	168-275
V3	N-terminal	175-275
V4	C-terminal	135-268
V5	C-terminal	135-262
V6	Internal	135-275 Δ218-234

Table 1.4: Suggested V antigen fragments which may retain protective properties. Adapted from Vernazza *et al.* (2009).

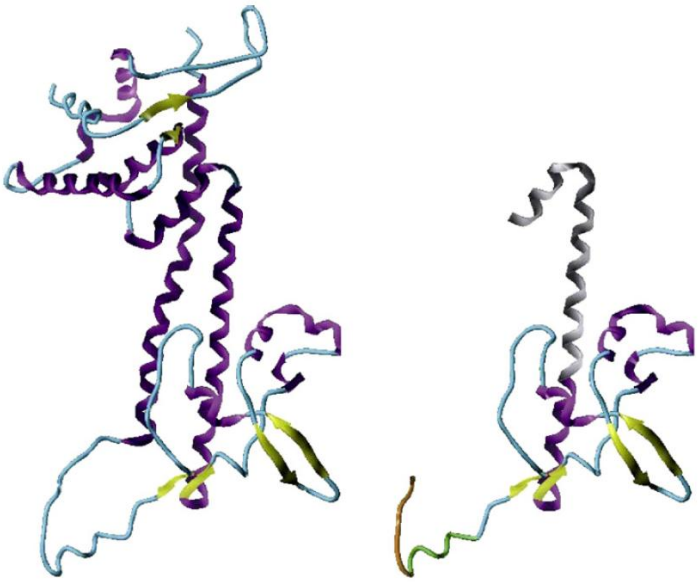


Figure 1.18: Ribbon-tube representation of modelled structures coloured by secondary structure: strands, yellow ribbon; helices, purple ribbon; loops, cyan blue. Left image: V antigen structure (a.a. 28-322). Right image: V antigen a.a. 135-275 region including a.a. 135-167 (deleted in V2 fragments and shown in grey), a.a. 269-275 (deleted in V4 fragment and shown in orange), a.a. 263-269 (deleted in V5 fragment and shown in green). Adapted from Vernazza *et al.* (2009).

1.6. Project aims

Despite the wealth of research on hunting different therapeutic interventions for sepsis, this medical conditions current therapy is limited to anti-infectives and supportive care. This project aimed to determine whether *Yersinia Pestis* V antigen has the ability to suppress sepsis associated inflammatory responses *in vitro* and *in vivo*. In order to elucidate this, both monocytic (THP-1) and macrophage (RAW 264.7) cells were used to test if *Yersinia pestis* V antigen is able to exhibit immunomodulatory activity that can treat or reverse the outcome of sepsis.

The objectives of this project were:

- Purify *Yersinia pestis* V antigen using affinity chromatography;
- Investigate the participation of pattern recognition receptors, in particular Toll-like receptors, Nod-like receptors and adaptor molecules, in response to LPS induced sepsis;
- Determine the effect of *Yersinia pestis* V antigen on pattern recognition receptors;
- Determine the inflammatory immune responses triggered by *Yersinia pestis* V antigen;
- Purify and Identify the function domain of *Yersinia pestis* V antigen;
- Determine the effects of *Yersinia pestis* V antigen fractions (V1, V3, V4 and V5) on innate immune signalling and cytokine secretion.
- Use confocal microscopy to elucidate whether *Yersinia pestis* V antigen associates with pattern recognition receptors, signalling molecules and cellular organelles;
- Finally, examine the ability of *Yersinia pestis* V antigen in suppressing sepsis associated inflammatory response *in vivo*.

Chapter 2

Materials and Methods

2.1. Antibodies

Primary antibodies	Species	Company
TLR 1	Mouse	Hycult
TLR 2	Goat	Santa Cruz Biotechnology
TLR 4	Goat	Santa Cruz Biotechnology
TLR 6	Goat	Santa Cruz Biotechnology
TLR 7	Goat	Santa Cruz Biotechnology
TLR 9	Goat	Santa Cruz Biotechnology
CD14	Rabbit	Santa Cruz Biotechnology
NOD 1	Goat	Santa Cruz Biotechnology
NOD 2	Mouse	Hycult
NLRP 1	Mouse	Hycult
NLRP 3	Rabbit	Santa Cruz Biotechnology
NLRP 12	Rabbit	Santa Cruz Biotechnology
Caspase-1 p10	Rabbit	Santa Cruz Biotechnology
Caspase-1 p20	Goat	Santa Cruz Biotechnology
Phospho-IκBα	Rabbit	New England BioLabs
Yersinia pestis V antigen	Mouse	Abcam
EEA1 (Endosome)	Goat	Santa Cruz Biotechnology
IRF-7	Goat	Santa Cruz Biotechnology
IRF-3	Goat	Santa Cruz Biotechnology
MyD88	Rabbit	Santa Cruz Biotechnology

Secondary antibodies	Label	Company
Goat anti-rabbit	FITC	Jackson ImmunoResearch
Rabbit anti-goat	FITC	Dako Cytomation
Rabbit anti-mouse	FITC	Dako Cytomation
Goat anti-mouse	FITC	Jackson ImmunoResearch
Goat anti-rabbit	HRP	Dako Cytomation
Goat anti-rabbit	Cy3	Jackson ImmunoResearch
Rabbit anti-goat	Cy5	Jackson ImmunoResearch
Donkey anti-goat	Alexa fluor 633	Invitrogen

Table 2.1: Primary and secondary antibodies used in this study. Secondary antibodies were conjugated with either fluorescent fluorochrome (Fluorescein isothiocyanate (FITC)),

Horseradish Peroxidase (HRP) for enhanced chemiluminescence (ECL), Cyanine 3 (Cy3), Alexa fluor 546 or Alexa fluor 633.

2.2. Cell lines

2.2.1. Tissue Culture

To ensure sterile conditions during tissue culture all procedures were performed in a Microflow Peroxide Class II Advanced Biological Safety Cabinet. Solutions, plasticware and glassware were autoclaved for tissue culture. Hood and equipment was decontaminated with 1% Aqueous Virkon (Antec International). A lab coat, disposable gloves and over-shoes were worn. Cell lines were grown at 37^{o}C in a 5% CO_2 humidified atmosphere.

2.2.2. Human monocytic leukemia cell line

Human monocytic leukemia (THP-1) cells were initially cultured in 25cm^2 flasks (Nunc) and then transferred into 80cm^2 flasks (Nunc). THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% Foetal Calf Serum (FCS (Biosera)) and 2% MEM Non-essential Amino Acid Solution (100X) (Invitrogen).

THP-1 cells were obtained from the European Collection of Animal Cell Cultures (ECACC). THP-1 cell line derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia.

THP-1 cells were passaged by re-suspending the suspension cells with fresh medium using a pipette. Cells were frozen in freezing medium consisting of 10% (v/v) dimethyl sulfoxide (DMSO) in FCS.

2.2.3. Mouse leukemic monocyte macrophage cell line

Mouse leukaemic monocyte macrophage (RAW 264.7) cells were cultured in 25cm^2 flasks (Nunc) in Dulbecco's Modified Eagle Medium (DMEM) 1000mg/L glucose medium (Invitrogen) supplemented with 10% FCS (Invitrogen) and 2% MEM Non-essential Amino Acid Solution (100X) (Invitrogen).

RAW 264.7 cells obtained from the European Collection of Animal Cell Cultures (ECACC). RAW 264.7 cell line derived from the ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV).

The proteolytic enzyme trypsin (Sigma) was used for detaching and passaging of RAW 264.7 cells. Cells were frozen in freezing medium consisting of 10% (v/v) DMSO in FCS.

2.2.4. Cryogenic preservation

Cryogenic preservation was used to maintain reserve cell lines. Cell lines were placed in cryotubes which then were placed in a -80ºC freezer for 24 hours and then into liquid nitrogen for long-term preservation. This step down in temperature allowed the cells to adjust to the liquid nitrogen.

Healthy population of cells were harvested by pipetting for suspension and semi-adherent cells, or by the use of the proteolytic enzyme trypsin (Sigma) for adhesive cells. Cells were placed in a 15ml tube (Nunc) and centrifuged at 12000RPM for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in 1.5ml of 10% (v/v) of freezing medium dimethyl sulfoxide (DMSO) in FCS. Cells in the freezing medium were then transferred into cryotubes (Nunc), and placed into an insulated container and then into the -80ºC freezer for 24 hours. After 24 hours the cryotubes were placed in a liquid nitrogen storage tank (-196ºC).

2.3. Cell counting

A hemocytometer is a cell counting glass microscope slide that allows one to obtain an approximation of the cell concentration (cells/ml) of a cell suspension. This device allows consistence throughout experiments when seeding cells.

This method of cell counting works on the basis of there being an exact known volume in the chamber of the hemocytometer (Figure 2.1). A quartz cover glass sits precisely 0.1mm above the chamber floor, each grid is 1x1mm, so the volume above each single grid is 0.1µl. By observing the chamber under the microscope it is possible to count the number of cells in cells/ml.

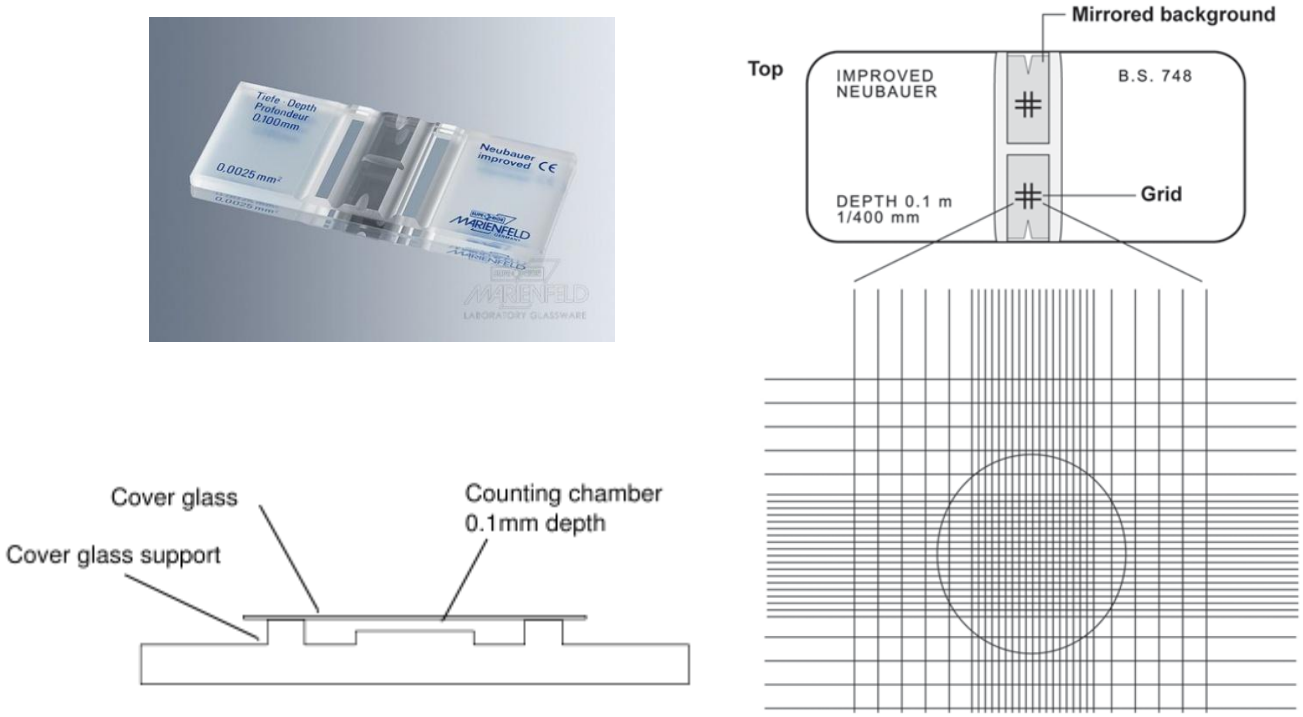


Figure 2.1: Hemocytometer, used to count cells in suspension. It works on the basis of there being an exact known volume in the chambers of the hemocytometer. A quartz cover glass sits precisely 0.1mm above the chamber floor, where each grid (highlighted by the circle) is 1x1mm, therefore the volume above each grid is 0.1µl. Adapted from: http://www.invitrogen.com/site/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/counting-cells-in-ahemacytometer.html / www.tekeven t.com/bm.pix/chamber-n-improved-brightline-with-v-06_500_30-2.s600x600.jpg (12/08/2013).

The number of cells in each grid in the counting area should be recorded and placed in the following equation:

$$\frac{Total\ number\ of\ cells}{Total\ number\ of\ grids\ used} \times 10^4 = cells/ml$$

2.3.1. Hemocytometer protocol

Cells were confluent when concentration was determined. Adherent cells were put in suspension by the use of the proteolytic enzyme trypsin whereas suspension and semi-adherent cells were re-suspended by agitation.

A few drops were added to the specialized Neubauer hemocytometer in order to flood its chambers. Cells were counted and recorded from the central and all corner grids were used. This data was entered into the equation in Section 2.3.

A cover slip was placed on a Neubauer hemocytometer. Cells were thoroughly re-suspended using a sterile Pasteur pipette and loaded on the edge of the cover slip, ensuring that both chambers of the Neubauer hemocytometer were flooded. The chambers were observed under a light microscope where cells were counted.

2.4. Viability test

Trypan blue is a diazo dye that is used to differentiate between live and dead cells. In this study, trypan blue was used as a viability test in order to determine the condition of cell populations before certain procedures.

The trypan blue viability test works on the basis that live cells have undamaged cell membranes that are highly selective in their up-take and thus will not allow the dye to

be absorbed by the cell. On the other hand, dead cells whose membranes have been compromised allow the trypan blue to enter the cell thus becoming stained.

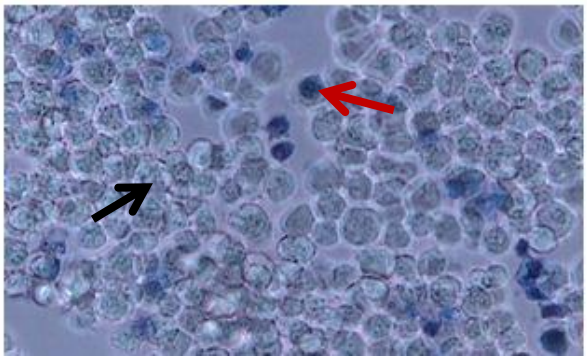


Figure 2.2: Trypan blue viability test. Live cells with intact cell membranes have not absorbed the dye therefore are viable (black arrow). Dead cells (red arrow) that have disrupted cell membranes allow the dye to enter resulting in a dark blue staining of the cell. Adapted from http://www.intechopen.com/source/html/19106/media/image5.jpeg (12/08/2013).

2.4.1. Trypan blue viability assay protocol

Confluent cells were washed with sterile X1 PBS and then enough trypan blue (Sigma) to cover the cells was added and left for 10 minutes. The cells were then washed with sterile X1 PBS and analysed using a Neubauer hemocytometer (see section 2.3).

2.5. V antigen

2.5.1. Recombinant LcrV plasmid (pVG110)

The recombinant LcrV plasmid (pVG110) with a glutathione S-transferase (GST) tag used in this study was kindly provided by Prof. Richard Titball (Chemical and Biological Defence Establishment, Porton Down, Salisbury, UK) as previously described (Carr et al., 1999). The plasmid was transformed into *E. coli* BL21 cells.

2.5.2. Fermentation of LcrV

E. coli BL21 cells containing the recombinant plasmid pVG110 was cultured in Luria broth containing 100mg/ml ampicillin (Fisher Scientific). Cultures were placed in a 37ºC rotary incubator at 120RPM until an A_{600} of 0.3 was achieved; cultures were then induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fisher Scientific). Cultures were incubated for a further 6 hours in a 37ºC rotary incubator at 120RPM. Cultures were then harvested at 7000RPM in a 4ºC centrifuge for 30 minutes.

2.5.3. Purification of LcrV

Once the broths had been centrifuged, the resulting pellets were resuspended in 5ml of X1 Phosphate buffered saline (PBS) and placed in 50ml tubes, the centrifuge container was washed out with a further 5ml X1 PBS and this was also added to the 50ml tube. The *E. coli* suspension was then frozen (-20ºC) and thawed 6 times. Tubes were vortexed vigorously between freezing and thawing. This ensures that the *E. coli* breaks and the recombinant protein can be collected. The tubes were then centrifuged at 3500RPM in a 4ºC centrifuge for 30 minutes. The supernatant was placed in a sterile 50ml tube and the resulting pellet was discarded. This process was repeated until the supernatant was clear. The supernatant was then purified using a Glutathione Sepharose (GST) purification column supplied by GE Healthcare.

2.5.3.1. Purification of V antigen fractions

In order to determine which region of V antigen is modulating innate response against LPS, V antigen fragments were generated. V antigen fractions were cloned in *E. coli* and kindly supplied to us by Dr. Claire Vernazza, Ministry of Defence, Porton Down. In order to purify the V antigen fractions, the *E. coli* strain had to be cultured in Luria broth and the protein isolated using affinity chromatography. V antigen fragments were selected according to previous studies performed by Vernazza et al. (2009) (Table 2.2).

Fragment	Deletion type	Amino acids
V1	-	135-275
V2	N-terminal	168-275
V3	N-terminal	175-275
V4	C-terminal	135-268
V5	C-terminal	135-262
V6	Internal	135-275 Δ218-234

Table 2.2: V antigen fragments. In this study the V antigen fragments selected were V1, V3, V4 and V5. Adapted from Vernazza et al. (2009).

2.5.4. Glutathione sepharose (GST) protein purification

GST tagged proteins can be purified from lysate using glutathione immobilized on a sepharose matrix via ligand specificity (figure 2.3). The GST tag has a high affinity for glutathione (GSH). Elution of bound GST fusion proteins from the column is achieved under mild, non-denaturing conditions (Wilson and Walker, 2005).

Attachment of the ligand (glutathione) to the affinity phase occurs via a spacer. The spacer allows optimum ligand interaction with the GST tagged protein.

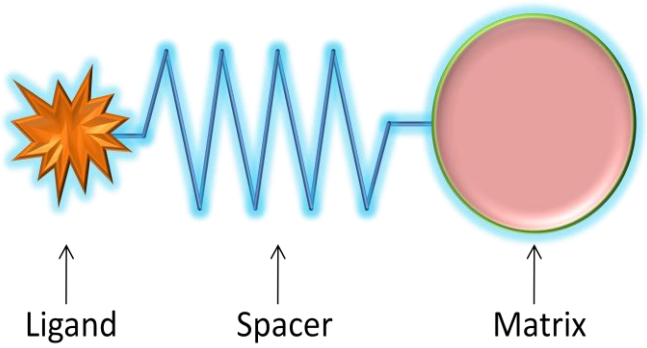


Figure 2.3: Matrix/spacer/ligand interaction. The glutathione ligand (orange) is coupled via a 10-carbon spacer (blue) to highly cross-linked 6% agarose (pink). The spacer and ligand can be manipulated to suit the requirements of the separation.

To elute the GST-tagged protein from the column an enzyme is used to cleave the GST from the protein of interest and thus leave the relatively large GST tag (~26KDa) bound to the affinity matrix. PreScission™ Protease (GE Healthcare) was used in this study. This protease is highly specific and also has a GST tag so on elution it remains bound to the matrix eliminating need for extra purification thus increasing the resolution and yield of the elute.

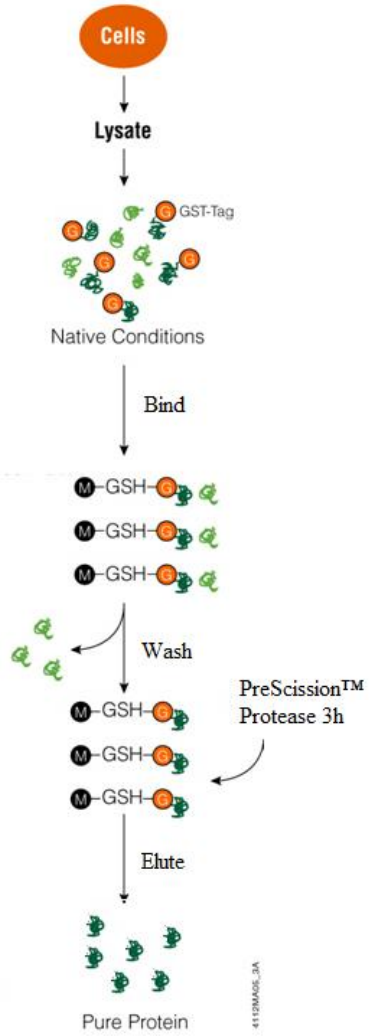


Figure 2.4: GST-tagged protein purification. The GST tag (orange) has a high affinity for glutathione (GSH) and binds the protein of choice (dark green) to the affinity matrix (black). Washing removes unwanted lysate (light green). PreScission^{TM} Protease (GE Healthcare) was used to cleave the GST tag from the protein leaving purified protein that does not require further purification. Adapted from source (Corporation, 2012).

2.5.5 LcrV-GST-tagged protein purification

The supernatant that was harvested from the LcrV fermentation was passed through a 10ml Glutathione Sepharose (GST) purification column (GE Healthcare). The column was equilibrated by passing through 10ml PBS/1% Triton. 10ml crude V antigen was then passed in the column matrix and the column was sealed to terminate flow. The column was left to incubate at room temperature for 1hr. After incubation the column

was washed out with 20ml 1X PBS and the elution was discarded. To the column 10ml cleavage buffer (50mM Tris/150mM NaCl/1mM EDTA/1mM DTT/0.01% (w/v) Triton X-100) was added. 150µl PreScission™ Protease (GE Healthcare) was then added and allowed to enter the matrix. The column was sealed to terminate flow. The column was then left to incubate for 3 hours at room temperature. After the incubation, 20ml cleavage buffer was used to wash out the column and the elution collected in a sterile bottle.

2.5.6. Protein concentration

In order to concentrate the purified proteins 10kDa cut off Centriprep centrifuge concentrators (Millipore) were utilised. Centriprep concentrators consist of two tubes, one housed in the other (Figure 2.5). The base of the internal tube has a permeable membrane that allows molecules through which are 10kDa or smaller, retaining the protein in the external tube. During centrifugation, the centrifugal force pushes anything that is ≤10kDa through the membrane into the internal tube. The sample remains in the exterior tube whilst the waste runs through the membrane into the internal tube, where it is then discarded.

Sterile 10kDa cut off Centriprep concentrators (Millipore) were filled to the “fill line” with purified sample. The concentrators were then placed in the centrifuge and spun at 2500RPM for 40 minutes at 4^{o}C. The content of the internal tube was discarded. The content of the exterior tube was placed back into the original elution and any precipitate re-suspended. This process was repeated until approximately 1-2ml of purified and concentrated protein remained. Sterile X1 PBS was used to perform buffer exchange on the protein sample. X1 PBS was filled to the “fill line” and the Centriprep spun at 2500RPM for 40 minutes at 4^{o}C. This was performed three times with an end sample of approximately 1-2ml. Protein concentration was determined by spectrophotometry.

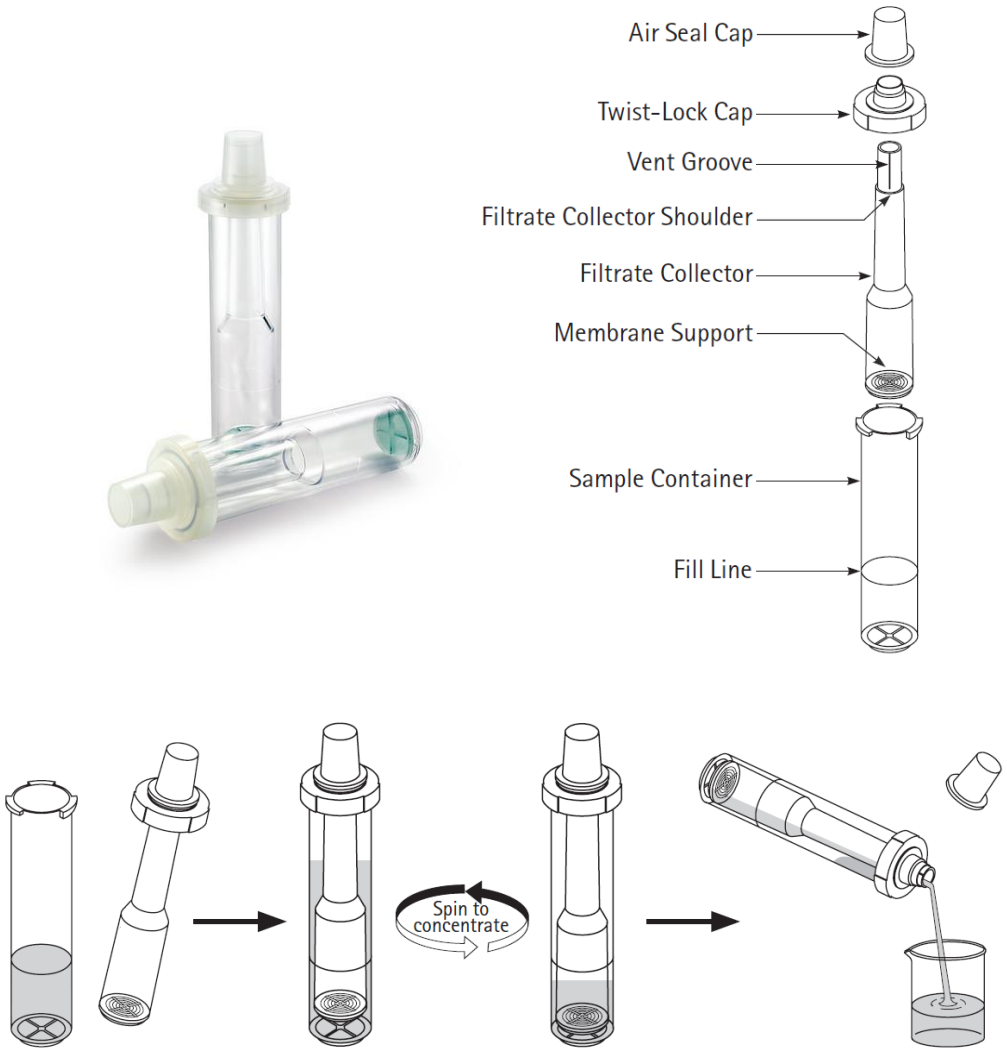


Figure 2.5: Centriprep concentrators. During centrifugation (2500RPM for 40 minutes at 4ºC) the centrifugal force pushes anything that is ≤10kDa from the sample container, through the cut off membrane, into the filtrate collector. Internal tube (filtrate collector) = waste. External tube (sample container)=concentrated sample.

2.5.7. Endotoxin removal

Bacterial endotoxin contamination has proven a big problem in research, especially in the field of immunology. When expressing a protein in an *E. coli* strain, for example, endotoxin from the *E. coli* can easily contaminate the purified product. Any cell stimulations performed with the contaminated protein will have a double effect of the protein itself and the contaminant, producing inconclusive data. To avoid this problem endotoxin can be removed by ultra-filtration, ion exchange chromatography, two phase

extraction or ligand specificity chromatography. In this study the Profos EndoTrap® blue 10 (Hycult Biotechnology) assay was used to remove endotoxin from purified protein samples, this assay utilizes ligand specificity chromatography (Hycult®Biotech, 2011).

Profos EndoTrap® blue is an affinity matrix that is designed to remove bacterial endotoxins from aqueous solutions, even at low endotoxin concentrations. Pre-packed columns have the EndoTrap blue ligand covalently bound to beaded agarose. The EndoTrap blue ligand is highly specific for bacterial endotoxin and has very low non-specific binding, giving high sample yields free of endotoxin (Hycult®Biotech, 2011).

2.5.7.1. Endotoxin removal protocol

Profos EndoTrap® blue 10 assay columns (Hycult Biotechnology) were firstly regenerated. X3 the column volume of regeneration buffer (Hycult Biotechnology) was passed through the column and the elution discarded, this step was repeated. X3 the column volume of equilibration buffer (Hycult Biotechnology) was passed through the column and the elution discarded, this step was repeated. X1 the column volume of sample was passed into the column, the elute was collected in sterile glassware. X3 the column volume of equilibration buffer was then passed through the column and collected in the same sterile glassware. Endotoxin free sample was re-concentrated in sterile Centriprep concentrators (Section 2.5.6) to a final volume of 1-2ml.

2.5.8. Endotoxin detection

The HyCult LAL Chromogenic Endotoxin Quantitation Kit was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* Amebocyte Lysate (LAL) assay. The LAL method for measuring endotoxin is based on the interaction of endotoxins with the proenzyme Factor C found in circulating amebocytes of the horseshoe crab *Limulus polyphemus*. The proteolytic activity of this proenzyme is activated in the presence of LPS. The Chromogenic *Limulus* Amebocyte Lysate

assay measures endotoxin levels by measuring the activity of this protease in the presence of a synthetic peptide substrate that releases p-nitroaniline (pNA) after proteolysis, producing a yellow colour that can be measured by reading the absorbance at 405nm (Hycult®Biotech, 2011).

To accurately measure endotoxin levels in a sample, the LAL assay uses an endotoxin standard of known concentration that is derived from *E. coli* strain O111:B4. This standard is provided with each kit and is used to create a standard curve. The endotoxin concentration is determined by extrapolating the absorbance of an unknown sample against this standard curve, similar to ELISA or total protein quantitation assays. Minimum concentration of LPS which can be measured is 0.01 EU/ml (1 EU = 100pg LPS).

Following endotoxin removal using the Endotrap columns, all purified recombinant proteins were tested for the presence of endotoxin using the LAL assay. It was shown that there were no detectable endotoxin in the purified samples.

2.5.9. Determination of protein concentration

After the Endotrap step and concentrating the sample, a UV spectrometer was used to determine the concentration of the sample by use of the formula shown below. The theory behind this formula is that the amino acid residues tyrosine and tryptophan within a protein show maximum absorption at a 280nm wavelength. While the proportion of these residues within a protein do vary and so do the extinction coefficients for the proteins, most proteins have an extinction coefficient within the range of 0.4-1.5. Following one of the Beer-Lambert law's equations ($Absorbance = exticticon\ coefficient \times concentration\ of\ solution \times length\ of\ solution$) a fair approximation of the protein concentration of a solution in a quartz cuvette with 1cm length can be calculated. However, there can often be contamination of nucleic acids which have an absorbance 10 times that of protein at this wavelength so even a small

amount of nucleic acids can greatly influence absorbance. The formula corrects for this possibility (up to 20% contamination with nucleic acids) by also measuring the sample's absorption at 260nm wavelength, the maximum absorbance of nucleic acids, multiplying that by a ratio determined by early researchers and subtracting that value from the one obtained from the absorbance at 280nm (Boyer, 2006, Wilson and Walker, 2005).

A UV spectrophotometer was used to determine the absorbance of the purified and concentrated V antigen solution at 260nm and 280nm. Then, concentration was calculated using the following formula:

$$[Protein](mg/ml) = 1.55 \times A_{280} - 0.76 \times A_{260}$$

2.6. Cell stimulations

Throughout this study all cell stimulations were carried out in their respective mediums (Section 2.2) and incubated at 37ºC in a 5% CO_2 humidified atmosphere. 1,000,000 cells per sample were stimulated during the course of this study. Ligand concentrations were kept constant throughout this study (Table 2.3).

Ligand	Concentration
Lipopolysaccharide (LPS)	100ng/ml
V antigen	50µg/ml
V1	50µg/ml
V3	50µg/ml
V4	50µg/ml
V5	50µg/ml

Table 2.3: Ligand concentrations used in this study. All concentrations were kept constant throughout.

2.6.1. 25cm^2 flask stimulation

Human monocytic leukemia (THP-1) cells were cultured in 80cm^2 flasks (Nunc) and stimulated in flasks with a surface area of 25cm^2 (Nunc). Sterile conditions were practiced throughout. THP-1 cells were counted using a hemocytometer (Section 2.3). The cell to volume ratio was rectified to 1,000,000 cells/ml. The cells were then stimulated.

Mouse leukaemic monocyte macrophage (RAW 264.7) cells were stimulated in flasks with a surface area of 25cm^2 (Nunc). Sterile conditions were practiced throughout. The growth medium was aspirated off and the cells were washed with 3ml of fresh medium. 2ml of medium was then added to the flask. The cells were then stimulated.

2.6.2. Lab-Tek^{TM} slide stimulation

For confocal microscopy mouse leukaemic monocyte macrophage (RAW 264.7) cells were grown and stimulated on 8 well glass slides (Nunc Lab-Tek^{TM} Chamber Slide^{TM} System). The growth medium was aspirated off and the cells were washed with 400µl of fresh medium. 150µl of growth medium was then added to each well. The cells were then stimulated.

2.7. Immunofluorescence

Immunofluorescence is a technique whereby specific molecules are labelled and visualised with fluorescent antibodies. Immunofluorescent labelling can be utilised to find the relative abundance and localisation of a chosen antigen. Fluorochromes that emit light of a set wavelength are conjugated to antibodies. Fluorescence is emitted from fluorochromes that become excited when exposed to a laser beam which results on the emission of light at a certain wavelength, which in turn can be imaged and/or quantified by a number of techniques. The development of fluorescent labelling as a research tool has proven invaluable.

Fluorescence is emitted from fluorochromes that become excited when exposed to a laser beam of the correct wavelength. Electrons in the fluorochrome move into a higher energy state when a photon from the laser is absorbed. The atoms are said to change from their “ground state” to an “excited state”. The emitted light is detected and can be used to quantify and determine the localisation of a chosen antigen.

There are a number of different fluorochromes available which have different excitation and emission wavelengths. Such availability of fluorochromes coupled with high specificity antibodies allows multiple labelling of molecules and/or structures in a single sample at any one time. The table below shows the different fluorochromes used throughout this study (Table 2.4).

Fluorochrome	Excitation λ	Emission λ	Colour
Fluorescein isothiocyanate (FITC)	495nm	519nm	Green
Phycoerythrin (PE)	547nm	572nm	Yellow
Cy3	553nm	556nm	Red
Cy5	650nm	667nm	Blue
Alexa fluor 633	632nm	647nm	Red

Table 2.4: Fluorochrome excitation, emission and observed colour of fluorochromes used in this study.

Immunofluorescence can be carried out through two main tests – direct and indirect.

2.7.1. Direct immunofluorescence

Direct immunofluorescence involves the direct binding of a fluorochrome conjugated primary antibody/ligand to the antigen of interest (Figure 2.6). A specific antigen will be detected by the antibody, and the fluorophore it is attached to can be subsequently detected via microscopy or flow cytometry.

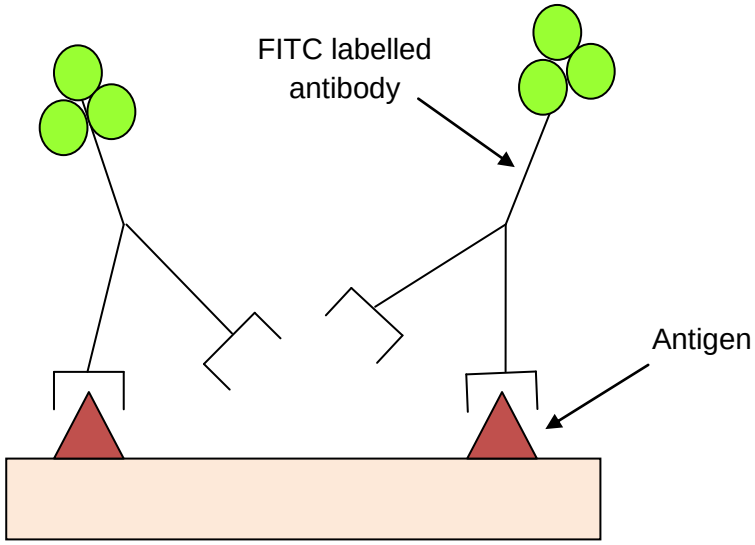


Figure 2.6: Direct immunofluorescence. Direct binding of fluorescein isothiocyanate (FITC) fluorochrome conjugated antibody to a target antigen. On excitation the labelled antibody emits fluorescence that can be detected and quantified.

When using direct immunofluorescence to label a protein in low abundance a weak signal may be achieved. To surpass this indirect immunofluorescence labelling can be used.

2.7.2. Indirect immunofluorescence

Indirect immunofluorescence differs from direct immunofluorescence in that it involves two compatible antibodies: an unlabelled primary antibody specific for the antigen of interest; and a fluorescently conjugated secondary antibody specific for the primary antibody (Figure 2.7). The structure of an antibody makes this possible, as it has two regions: a Fc region (fragment crystallisable region) and a Fab region (fragment antigen-binding region). The Fab region contains variable sections that determine which antigen is bound, whilst the Fc region is constant in a class of the same species. Antibodies can be designed in such a way that they contain the same Fc region but different Fab regions. In this way, primary antibodies with the same Fc region can be used to detect various antigens (due to differing Fab regions), and still be detected by a

single fluorescently-conjugated secondary antibody specific for the Fc region of the primary antibody.

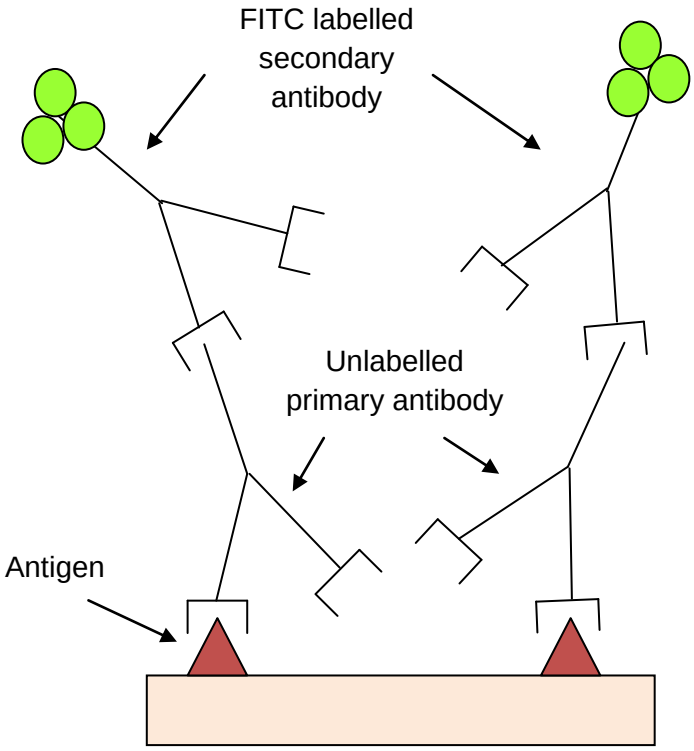


Figure 2.7: Indirect Immunofluorescence. Indirect immunofluorescence involves the binding of an unlabelled primary antibody to a chosen receptor, then the further binding of a fluorescein isothiocyanate (FITC) conjugated secondary antibody to the primary antibody. On excitation the labelled secondary antibody emits fluorescence that can be detected and quantified.

The primary and secondary antibody binding is species-specific. If a goat TLR4 primary antibody was used for example, an anti-goat fluorochrome conjugated secondary antibody will have to be used with this, such as a rabbit anti-goat antibody.

Indirect immunofluorescence has the advantage of being more sensitive than direct immunofluorescence because more than one secondary antibody can bind to any one primary antibody, thus giving a stronger signal.

2.8. Flow cytometry

Flow cytometry is a powerful technique used to analyse cellular characteristics of individual cells in a heterogeneous population. In this study, fluorescently labelled cells/cytokines were analysed using a Becton Dickinson (BD) Fluorescent Activated Cell Sorting (FACSCalibur^{TM}) system with software supplied by Cell Quest (Figure 2.8).

Figure 2.8: Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur^{TM}). The flow cytometer can quantify and distinguish between cells according to their fluorescence and physiological structure. http://medschool.creighton.edu/fileadmin/user/medicine/images/teachResearchPatient/Research/Flow_Cytometry_Core_Files/Images/Calibur2.jpg (07/11/2013).

Fluorescently labelled cells that pass through the FACS are each individually exposed to a laser with a single wavelength of light. Fluorescein isothiocyanate (FITC) conjugated antibodies were used in this study to label specific receptors. The resulting emitted light from the sample passes through a number of different detectors and analysed through the software supplied. Cytokines are labelled in much the same way using a cytometric bead array (CBA) kit allowing the determination of their concentration in a sample.

2.8.1. FACS System

FACS analysis can be divided into three main stages: fluidics, optics and signalling processing.

2.8.1.1. FACS fluidics

FACS analysis requires the cells to be individually analysed. When the cytometer is set to acquire, cells in a sample run through the chamber at a very high speed in a single file. This is achieved by the fluidics system of the FACS machine via a process known as hydrodynamic focusing (Figure 2.9). Hydrodynamic focusing is achieved when the sample of cells from a central core injector merges with a flowing stream of sheath fluid (saline solution) in an outer core, which is flowing at a much higher velocity in the same direction.

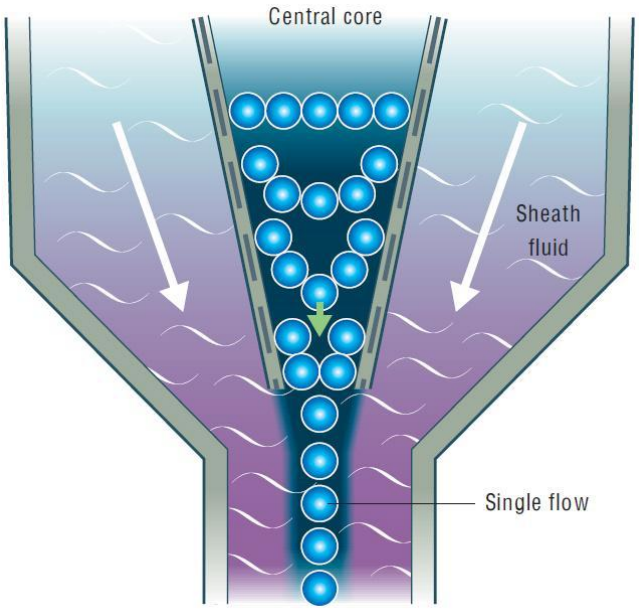


Figure 2.9: Hydrodynamic focusing of a sample by the fluidics system of the Fluorescence Activated Cell Sorter (FACS). The sample is injected from the central core into sheath fluid from the outer core which is flowing at a much higher velocity in the same direction. This compresses the cells in to flow in a single file for analysis. http://www.assay-protocol.com/uploads/Flow%20cytometry.JPG (13/11/2013).

Hydrodynamic focusing results in the cells entering the flow cell in single file to be exposed to the optics system.

2.8.1.2. FACS optics

As thousands of cells pass per second through the flow chamber they are subjected to one or more laser beams that have a programmed wavelength. When the laser strikes a cell, two forms of scattered light occur: forward scatter (FSC), which is the amount of light that is scattered in the forward direction, quantifies a cell's size and can distinguish between live cells and debris, this is detected at 20º off the excitation laser bead; and side scatter (SSC), which is the rest of the light collected on a detector 90º from the laser beam, shows the granularity of a cell. Fluorescence can also be detected. Lasers excite the fluorophores, and the subsequent fluorescent emissions travels along the same tour as the SSC signal (Figure 2.10).

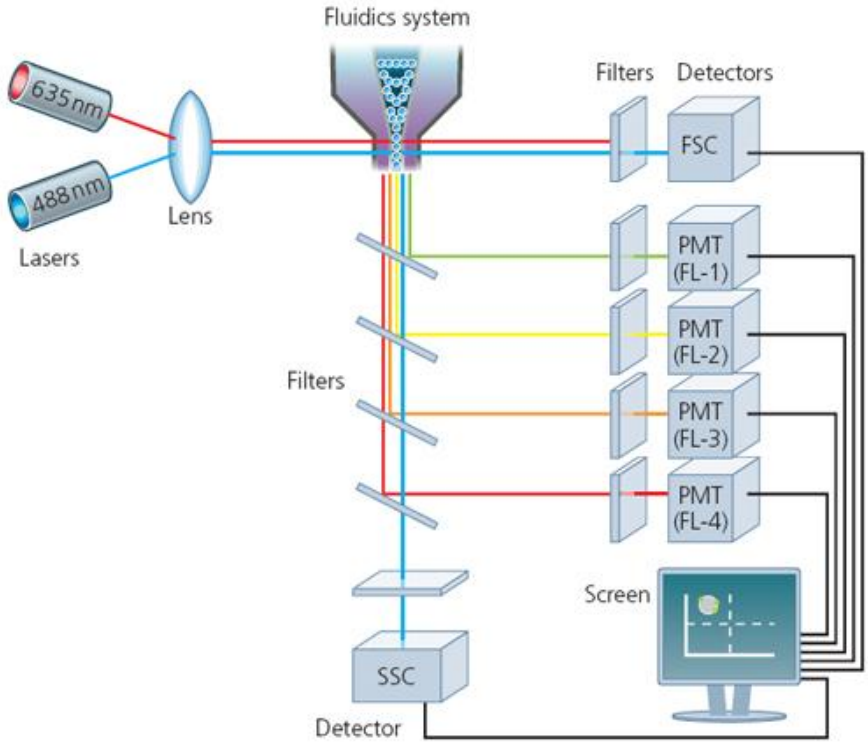


Figure 2.10: Arrangement of filters and detectors in the Fluorescence activated cell sorter (FACS) optics system. Fluorescent detection and side scatter (SSC) is achieved at 90º to the path of excitation laser beam. Forward scatter (FSC) is detected at 20º of the excitation laser beam. http://static.abdserotec.com/thumbnails/signal1.jpg (13/11/2013).

FSC and SSC uncover the cells morphology whilst fluorochrome emission is used to quantify a labelled antigen.

2.8.1.3. FACS signal processing

Signal processing was performed by the Cell Quest software. This software has complete control over the FACS allowing parameter to be tailored for each experiment. Photons sensed by the photomultiplier (PMT) detectors (Figure 2.10) are transformed to voltage and then relayed to the software. The combined information from the detectors is quantified, saved and displayed on the screen.

2.8.2. FACS application

Fluorescently labelled cells/cytokines were analysed using a Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur^{TM}) with software supplied by Cell Quest.

2.8.2.1. Pattern recognition receptor expression levels

Indirect immunofluorescence followed by flow cytometry was used to investigate the expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3, NLRP12 on THP-1 and RAW 264.7 cells in response to LPS, V antigen and its fractions V1, V3, V4 and V5.

Confluent cells were stimulated with 100ng/ml of lipopolysaccharide (LPS) and incubated for 1, 2, 4 or 6 hours. Some of these cells were either pre-incubated for 1 hour or post-incubated for 1, 2, 4 or 6 hours with 50µg/ml of V antigen, V1, V3, V4 or V5.

To perform the indirect immunofluorescence assay, after the incubation period, cell samples in eppendorf tubes were centrifuged at 12000RPM for 5 minutes at room temperature, the supernatant was aspirated off and the cells were re-suspended in X1

phosphate buffered saline (PBS). The tubes were centrifuged for 2 minutes at 13000RPM and the supernatant was aspirated. The cells were then fixed by adding 300µl of 4% paraformaldehyde (PFA) (Sigma), resuspended and left to incubate at room temperature for 15 minutes. PFA is used as a fixative, by cross-linking proteins, mainly the residues of the amino acid lysine, to lend rigidity to the cells and prevent further biochemical reactions. Cells were then washed twice with 500µl PBS/0.02%$_{(w/v)}$ bovine serum albumin (BSA) (Sigma)/0.02%$_{(w/v)}$ sodium azide (NaN_3) (Sigma)/0.02%$_{(w/v)}$ saponin (Sigma). BSA is used as a carrier protein to antibodies and as a general protein blocking agent. NaN_3 prevents the internalisation of surface antigens, which could produce a loss of fluorescent intensity. The amphipathic nature of Saponin makes it act as a surfactant, enhancing the penetration of proteins through the cell membrane. The samples were then centrifuged and the supernatant aspirated off again, followed by re-suspension in 100µl PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin. To this, 2µl of primary antibody was added and incubated at room temperature for 4 hours and left over night at 4ºC. After incubation the cells were washed two times. The pellet was then re-suspended in 100µl PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin, followed by incubation with 2µl of the respective secondary antibody for 45 minutes in the dark (to prevent photobleaching) at room temperature. The cells were then washed twice in PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin, then re-suspended in 500µl X1 PBS and transferred to Falcon flow tubes and analysed using a Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur^{TM}) with software supplied by Cell Quest. 10,000 cells not gated were analysed for each sample.

2.8.2.2. Cytokine analysis

To quantify the inflammatory response of THP-1 and RAW 264.7 cells the presence and levels of released cytokines were determined. Cytokines are the messengers that co-ordinate inflammation and give a direct representation of cellular response.

In this study the Human Inflammation BD™ Cytometric Bead Array (CBA) (BD Biosciences) kit was used to measure cytokine concentrations. The Human Inflammation BD™ CBA kit is capable of detecting six cytokines that play major roles in the human inflammatory response. These are Interlukin-8 (IL-8), Interlukin-1β (IL-1β), Interlukin-6 (IL-6), Interlukin-10 (IL-10), Tumor Necrosis Factor (TNF) and Interlukin-12p70 (IL-12p70). CBA analysis allows the fast and highly sensitive quantification of an array of cytokines in any one sample.

In order to bind cytokines present in a sample the beads are coated with capture antibodies specific for a particular cytokine. In the Human Inflammation BD™ CBA kit there are six bead populations with different fluorescent intensities specific for IL8, IL-1β, IL-6, IL-10, TNF and IL-12p70. Once the beads have bound to the respective cytokines a Phycoerythrin (PE)-conjugated detection antibody mix is added, this is a mixture of PE-conjugated antibodies specific for each bead (Figure 2.11).

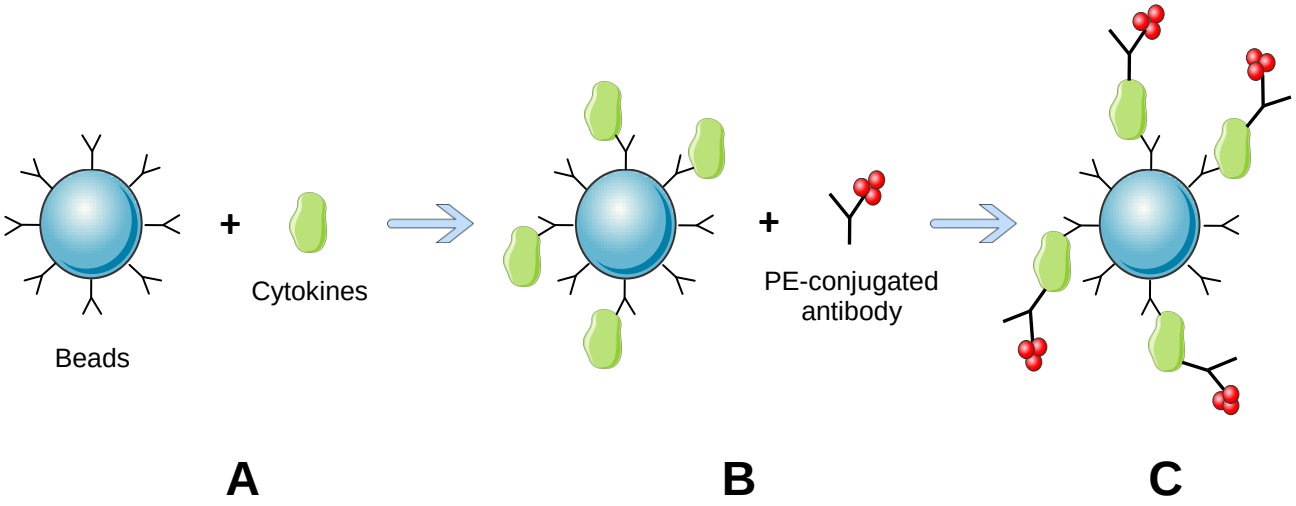


Figure 2.11: BD™ Cytometric bead array (CBA) system. A) CBA beads (blue) bind cytokines (green) in sample. B) Phycoerythrin (PE)-conjugated detection antibody is added. C) Cytokine can be seen sandwiched between the bead and the PE detection antibody.

The Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur™) was used in conjunction with Cell Quest software (Becton Dickinson) in order to run the samples.

CBA Analysis Software (Becton Dickinson) was used to process the raw data to cytokine concentration (pg/ml) using a previously calibrated curve.

2.8.2.2.1. CBA protocol

For each sample 20µl of bead mixture containing equal volumes of IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70 beads was added to a Falcon flow tube. To this 20µl of sample was added and the mixture vortexed briefly. 20µl of phycoerythrin (PE)-conjugated detection antibody mixture was then added and again vortexed briefly. The samples incubated for 3 hours at room temperature being left in the dark (to avoid photobleaching), gently shaking every 30 minutes. 1ml X1 PBS was then added to each sample which was then centrifuged at 1200RPM for 5 minutes. The supernatant was poured off leaving the pellet undisturbed. 300µl wash buffer (BD Biosciences) was added to each tube and then vortexed very briefly. The samples were assayed using the Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur^{TM}) used in conjunction with Cell Quest software (Becton Dickinson). The cytokine concentration (pg/ml) was determined by data processing CBA Analysis Software (Becton Dickinson).

2.9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used for the separation of proteins according to their Molecular Weight (MW). This method is used for several different purposes such as to identify a protein, determine protein size and abundance, find the number of proteins present and find the purity present of a sample.

The proteins in a sample will have a number of differences including length, shape, weight and charge. For SDS-PAGE to work correctly the proteins must be put in their native state, i.e. primary structure. SDS is an anionic detergent that disrupts the hydrogen bonds and Van der Waal's interactions in proteins and breaks down cell membranes. This destroys the proteins secondary, tertiary and quaternary structures causing them to become linear. SDS also gives the protein a uniform negative charge making the protein travel to the positive pole in an electrical field. The denaturation of the protein into its primary state was caused by heating the sample in a buffer containing β-mercaptoethanol.

The separation of the proteins occurs in the polyacrylamide gel which is made up of many acrylamide monomers that create a sieve like matrix where the proteins have to pass. The fact that the proteins are all denatured and negatively charged they will move in an electrical field towards the positive pole at the same rate. The cross-linked matrix makes it difficult for the molecules to move through the polyacrylamide gel, the smaller proteins have an advantage due to their size and thus move furthest the gel toward the positive terminal (Figure 2.12).

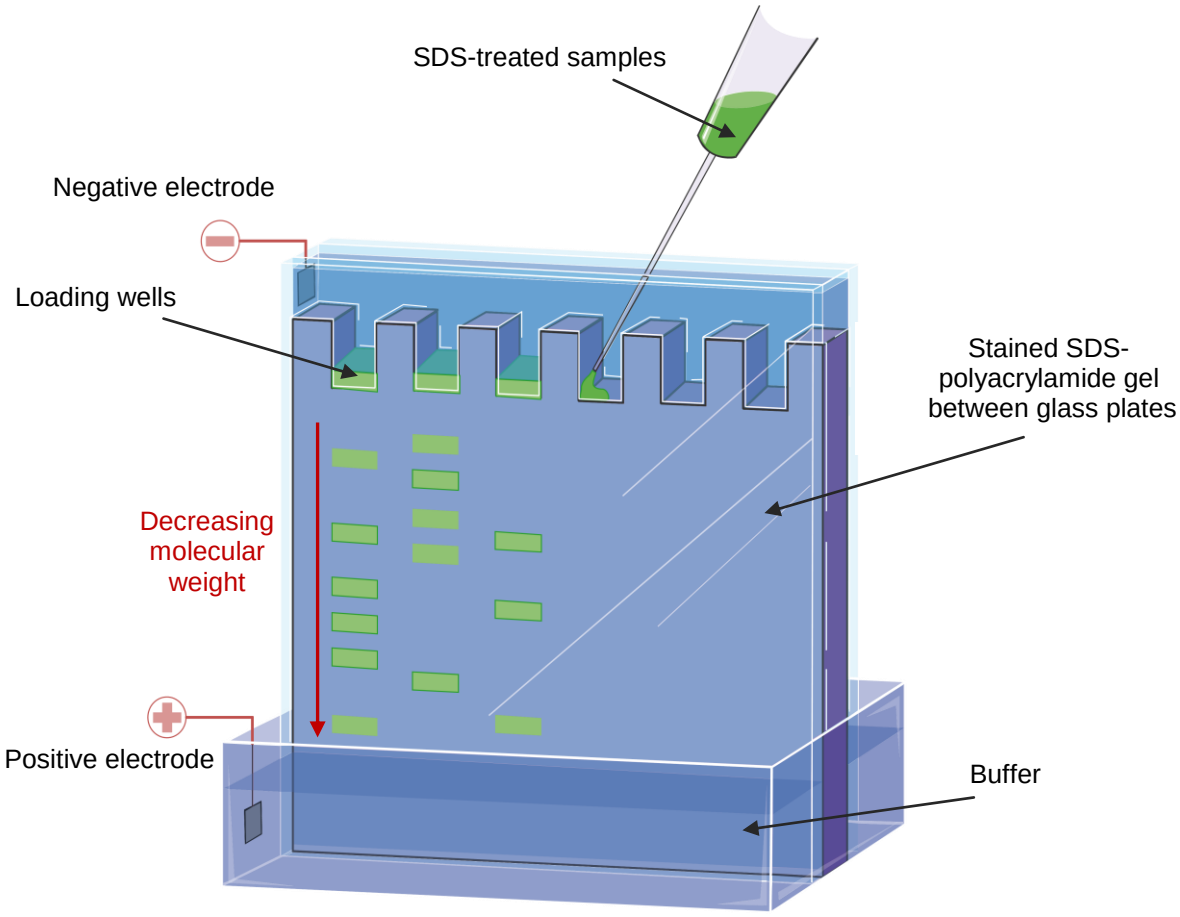


Figure 2.12: SDS-PAGE apparatus. SDS-PAGE is a technique for separating proteins in relation to their molecular weight. The application of voltage pulls negatively charged denatured proteins through the cross linked polyacrylamide gel matrix and separates them according to molecular weight.

Pore size within the matrix can be manipulated by varying the concentration of Acrylamide/Bis in a gel matrix. In this study a 10% Acrylamide/Bis was used. The gel is made up in liquid form and so it can be easily poured into the appropriate casts to set. The polymerisation of the gel is caused by Ammonium Persulphate (APS) and N, N, N', N'-tetramethylethylenediamine (TEMED) as they act as catalysts. Once these chemicals are added the gel forms cross links creating the sieve like matrix required.

For SDS-PAGE analysis in this study the Mini-PROTEAN 3 apparatus (BioRad) was utilised (Figure 2.13).

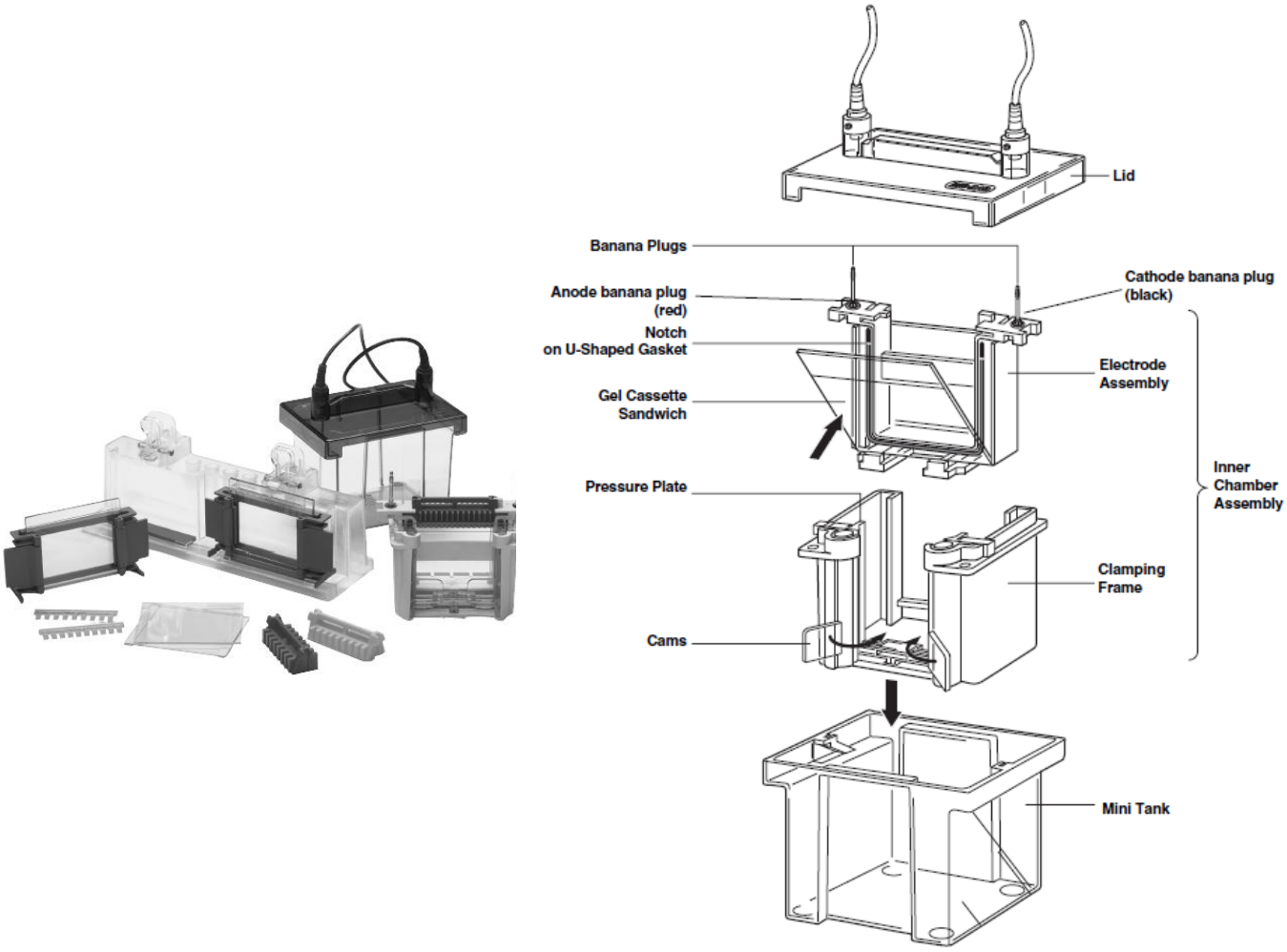


Figure 2.13: The Mini-PROTEAN 3 apparatus (BioRad) was used to run Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). http://www.plant.uoguelph.ca/research/homepage/raizada/Equipment/RaizadaWebEquipmentPDFs/9B.miniprotean3cellmanual.pdf (27/11/2013).

2.9.1. Casting SDS-PAGE gels

Gels were cast in 1mm width casts. Once the apparatus was set up, the resolving gel (1.5M Tris-HCL, 10% SDS, Acrylamide/Bis, 10% APS, TEMED and dH_2O) mixture was made up leaving the addition of APS (Sigma) and TEMED (Sigma) until last. Once the APS and TEMED were added the liquid gel was immediately poured (as they catalyse the polymerisation of the acrylamide gel) into the cast leaving adequate room for the teeth of the comb. On top of this, a thin strip of isobutanol was added in order to remove any bubbles and ensure that the gel surface was smooth and flat. The gel was

left at room temperature for 45-60 minutes to polymerise. Once the resolving gel was set, the isobutanol was poured off and then the gel was rinsed with dH_2O. The 4% stacking gel (0.5M Tris-HCL, 10% SDS, Acrylamide/Bis, 10% APS, TEMED and dH_2O) mixture was made up (again, 10% APS and TEMED were added once the gel was ready to be poured). This was then placed on top of the set resolving gel. A 1mm ten teethed comb was placed in the stacking gel mixture to create wells for the samples. The gel was left to polymerise at room temperature for 45-60 minutes.

Once the stacking gel had set, the comb was removed, the plates were unclipped from the casting stand and were placed, short plate facing inwards, into the electrophoresis tank (BioRad). X1 running buffer solution was added to the tank and filled between the plates.

2.9.2. Sample preparation for SDS-PAGE

To prepare the samples for running on the gel, X2 SDS-PAGE Reducing Sample Buffer was added to THP-1 and RAW 264.7 cells that had been incubated with LPS and either pre or post-incubated with V antigen or its fractions V1, V3, V4 and V5. Cells were left for 2 hours on the work top shaker and hen frozen at -20ºC for a minimum of one night. The SDS and the β-mercaptoethanol present in the reducing sample buffer denature the proteins disrupting secondary, tertiary and quaternary structures. The glycerol helps preserve the proteins at low temperatures, and weighs down the samples when loading them into the wells. The Tris present acts as a buffer (Tris has an effective pH range between 7 and 9.2). The addition of Brompthenol Blue allows the samples to be visualised as they pass through the gel.

2.9.3. Running samples

100µl of each sample was added to separate eppendorfs and boiled for 10 minutes, along with a molecular weight marker (2µl biotinylated SDS-PAGE broad range standards (BioRad) in 40µl SDS-PAGE Reducing Sample Buffer), to help denature the

proteins further. The standards are a mixture of biotinylated proteins with consistent molecular weights, allowing for accurate molecular weight determination of immune detected proteins. Once let to cool for approximately 5 minutes, 40µl of the samples (10µl of the standards) were then loaded into their respective wells in the polyacrylamide gel. The gel was run at a constant voltage of 200V for 45 minutes, or until the blue dye ran off the bottom of the gel.

2.9.4. Coomassie blue staining of SDS-PAGE gel

Coomassie blue stain binds non-specifically to nearly all proteins. It does so through Van der Waal's force interactions with amino acids such as histadine, arginine, lysine and tyrosine. Coomassie blue staining is used as protein concentration determination (through the Bradford protein assay) or visualization of protein bands on SDS-PAGE gels. In this study Coomassie blue staining was used to visualize protein bands separated in SDS-PAGE gels.

2.9.4.1. Coomassie blue protocol

The SDS-PAGE gel was removed from the electrophoresis glass plates and placed in fixing solution overnight. The gel was then removed from the fixing solution and submerged in Coomassie blue stain for 60 minutes. The Coomassie blue was removed and the gel was washed with de-stain. Every 10 minutes the de-stain was changed until stained protein bands could be observed and the background was clear.

2.10. Western blot

Western blotting is a technique used to transfer the proteins that have been separated by SDS-PAGE onto a nitrocellulose membrane in the same formation as they were in the gel. Following SDS-PAGE the gel is removed and undergoes an electroblot where a sandwich of the gel (with the stacking gel removed) and the nitrocellulose transfer membrane (Whatman Protran) compressed in a cassette between two layers of blotting

paper and sponge like pads pre-soaked in transfer buffer (Figure 2.14). The gel transfer mechanism uses the same method as SDS-PAGE to pull proteins out of the polyacrylamide gel matrix and into the nitrocellulose paper. A constant current of 210mA was applied for 60 minutes, electrophoretically transferring the proteins from the gel to the membrane.

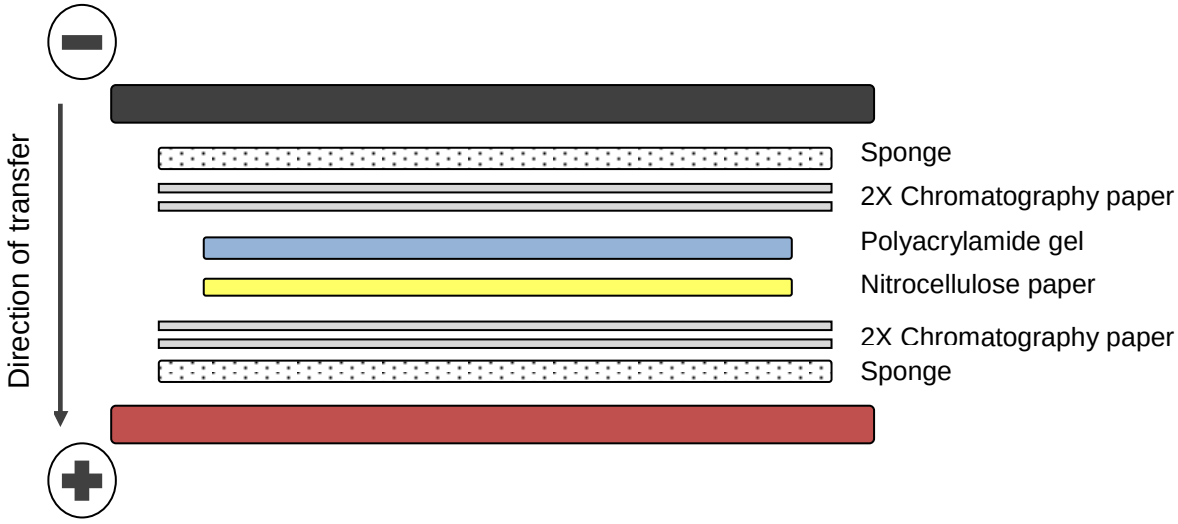


Figure 2.14: Electroblotting layout. Electroblotting is used to pull proteins from a polyacrylamide gel onto a nitrocellulose membrane. The direction of protein movement is from negative to positive (black arrow). The polyacrylamide gel and the nitrocellulose gel are sandwiched between 2 sponges and 4 chromatography papers. The protein is pulled out of the polyacrylamide gel (blue) into the nitrocellulose membrane (yellow).

Once in the membrane the sample can be analysed through several methods: fluorescent detection, radioactive detection, colorimetric detection and chemiluminescence. These techniques allow one to view the position and relative concentration of a specific protein.

2.10.1 Blocking and labelling the nitrocellulose membrane

After the transfer was complete, the nitrocellulose membrane was removed and washed twice with X1 PBS-Tween. All washes and incubations were performed at room temperature on a rocking table, to ensure the membrane was fully covered and never left out to dry. The membrane was then blocked for 60 minutes using a blocking

buffer. The blocking buffer blocks any remaining hydrophobic binding sites, preventing the binding of primary antibody to the membrane itself and thereby reducing background signal. The membrane was then washed for 60 minutes in X1 PBS-Tween with two rinses every 15 minutes. The membrane was then placed in a low volume container and was submerged in 1:1000 dilution of the primary antibody (either Phospho-IκBα rabbit (New England BioLabs) or Caspase p-10 rabbit (Santa Cruz Biotechnology) in X1 PBS-Tween. The membrane was left on rotary at room temperature for 4 hours and then left in the fridge overnight. The next day the membrane was washed for 30 minutes in X1 PBS-Tween with two rinses every 15 minutes. The membrane was then placed in a low volume container and submerged in 1:2000 goat anti-rabbit Horseradish Peroxidase (HRP) secondary antibody (DAKO Cytomation) in X1 PBS-Tween. The membrane was left on rotary at room temperature for 45 minutes. With regards to the standards the membrane was incubated for 30 minutes with Streptavidin-HRP conjugate (GE Healthcare) at a 1:1500 dilution. The membrane was then washed for 3 hours in X1 PBS-Tween with two rinses every 15 minutes. This ensured all excess antibodies had been removed before visualising the bands using enhanced chemiluminescence.

2.11. Enhanced chemiluminescence

The emission of light as a result of the dissipation of energy form a substance in an excited state, caused by a chemical reaction, is called chemiluminescence. Enhanced chemiluminescence is a very sensitive and fast procedure which utilises light emitted from an enzymatic reaction to image a protein of choice. The exposure of a luminal based substrate (ECL reagents) to the HRP label causes a light emitting reaction that can be visualized using a high performance chemiluminescence film (Figure 2.15.).

Detection of the bands was performed in a dark room, to avoid exposing the chemiluminescence film to light. Excess PBS-Tween was drained from the membrane

before placing it (protein side up) on a Saran wrap. ECL western blotting reagents 1 and 2 were mixed in a 1:1 ratio (2ml total per membrane) added to the membrane, and incubated at room temperature for 1 minute. Excess reagent was dabbed off, the membrane was transferred to a fresh piece of Saran wrap, turned over (protein side down), and wrapped up ensuring that it was smooth and there was no bubbles. The membrane was then placed in a developing cassette where a sheet of high performance chemiluminescence film (Hyperfilm^{TM} from GE Healthcare) was placed on top of the membrane. The developing cassette was closed and an initial exposure of 2 minutes was performed. The film was developed, fixed and analysed. Depending on the intensity and clarity of the bands seen, the exposure time was optimised where a new sheet of chemiluminescence film would be exposed if necessary.

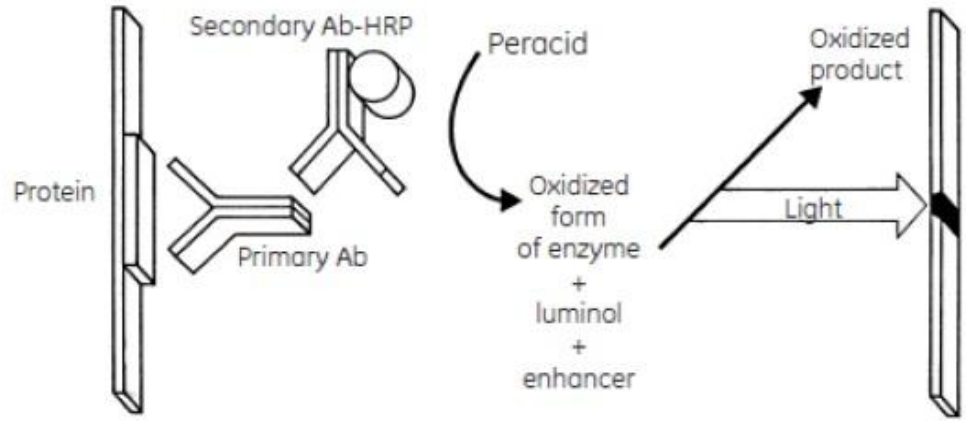


Figure 2.15: Enhanced chemiluminescence (ECL). Indirect labelling. The nitrocellulose membrane was labelled with a primary antibody and a horseradish peroxidase (HRP) conjugated secondary antibody. HRP catalyzes the oxidation of luminal to its excited state and light is emitted and detected on film. Adapted from http://openwetware.org/wiki/Image:ECL_Method.jpg (09/01/2014).

2.11.1. Stripping and reprobing nitrocellulose membranes

A membrane may be stripped of its bound primary and secondary antibodies, in order to reprobe it with different antibodies. The membrane was placed in stripping buffer and incubated for 4 minutes at 37ºC in a shaker incubator. After washing at room temperature with X1 PBS-Tween 3 times for 10 minutes each, the membrane was blocked for 60 minutes using 5% blocking agent, and washed twice for 15 minutes with X1 PBS-Tween. A different primary antibody can then be added, following the procedure in section 2.10.1.

2.12. Confocal microscopy

Confocal microscopy is a technique used to visualise the interactions and location of different proteins within cells. This system of imaging was originally developed in 1955 by Marvin Minsky. Confocal microscopy can create sharp images with very high spatial and temporal resolution that are far better than light or standard fluorescence microscopy.

Conventional microscopes function by exposing the whole sample to light and recording the light emitted. In this imaging technique the image produced includes in focus and out-of-focus light emitted of the specimen, from above and below the focal plane. This results in a blurred image where focused light is overlapped with unfocused light. A confocal microscope can create sharp images of specimens by excluding out-of-focus light in specimens which are thicker than the focal plane by using a spatial pinhole. This increases the micrograph contrast and enables reconstruction of three-dimensional images.

The confocal microscope uses two lenses to focus light from the focal point of one lens, through a pinhole aperture where the light is detected. This aperture can be controlled to cut out any light outside the focal plane (red light path in Figure 2.16).

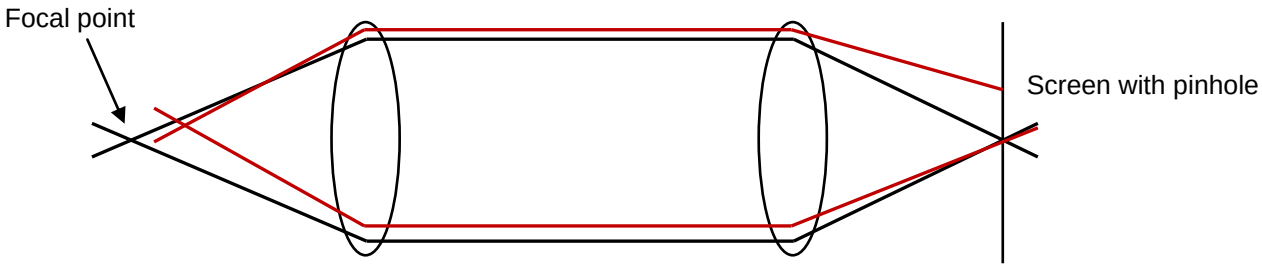


Figure 2.16: Lens layout of a confocal microscope. Confocal microscope uses two lenses to focus light from the focal point of one lens to a pinhole where the light is then detected. This method sieves out-of-focus light eliminating unwanted noise to produce a high resolution image.

The confocal microscope has the ability to reconstruct three dimensional images. It does this by taking multiple images of the sample through the microscope's Z axis. This allows one to stack the images together (Z-stack) to create a revealing three dimensional representation of the sample.

A confocal microscope works by scanning one or more focused beams of light, usually from a laser or arc-discharge source, across the specimen to create illumination. The laser beam passes through the microscope to the sample via a dichroic mirror and two motorised rotating mirrors. The motorised mirrors scan the laser beam over the sample. The light emitted is focused by two lenses, back in the direction of the original laser path, through a pinhole aperture and onto a photomultiplier tube (PMT) detector (Figure 2.17). The images collected from scanning the sample are processed by computer software into a two dimensional image.

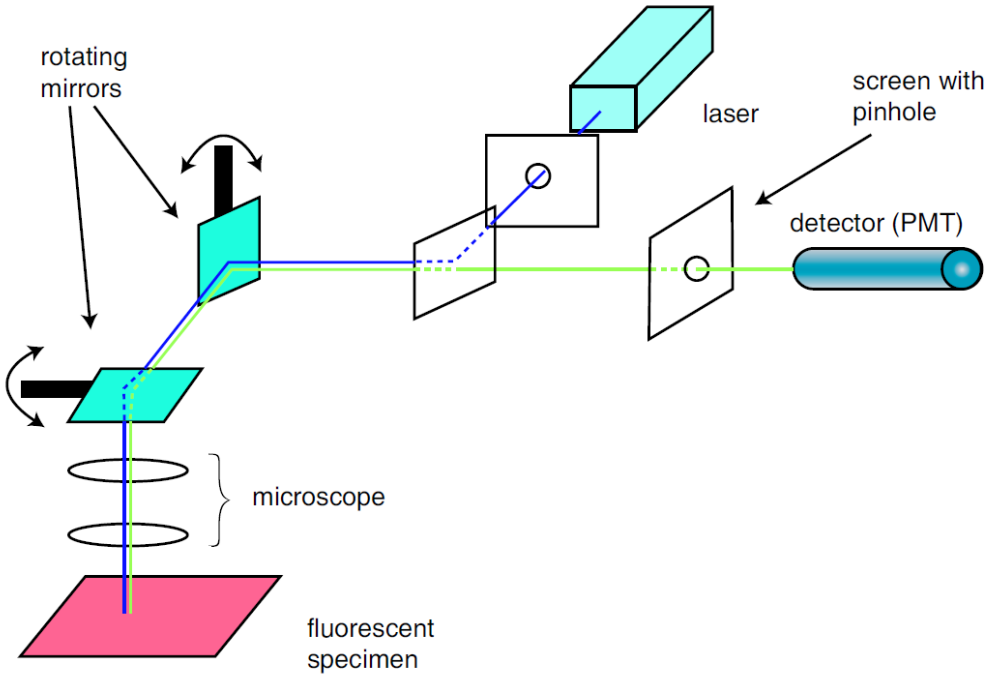


Figure 2.17: Light path in a confocal microscope. The path of the laser (blue) as it is reflected off the dichroic mirror and then the rotating mirrors. This is then focused onto the specimen via the microscope lenses. The green line indicates the path of light emitted by the fluorescent label in the specimen, as it passes back in the direction of the laser through a pinhole, ridding of any undesired light, and onto a photomultiplier (PMT) detector. Adapted from http://www.physics.emory.edu/~weeks/lab/papers/ebbe05.pdf (14/01/2014).

One of the great advantages of using a confocal microscope is that in spite of a relatively weak signal it is able to produce high quality images. It does so with the use of lasers and PMT detectors. The laser produces bright, low-divergent light that can be focused and collected with minimal light loss and the PMT detectors amplify the weakened signal.

In this study the samples analysed with confocal imaging were immunolabelled with fluorochromes via direct or indirect immunofluorescence (Section 2.7.). Fluorescence is emitted from the fluorochrome that becomes excited when exposed to a laser beam of the correct wavelength. The confocal microscope is capable of distinguishing and filtering between different emission wavelengths which enables multiple labelling of molecules and/or structures of interest.

In this study the confocal microscope utilised was a Carl Zeiss LSM 510 META using a 63x /1.4 Oil DIC Zeiss objective. The images were analysed using a Carl Zeiss LSM Image Browser software (Carl Zeiss, Inc). Cells were cultured on 8 well glass slides (Nunc Lab-Tek^{TM} Chamber Slide^{TM} System) which were labelled via direct and/or indirect immunofluorescence.

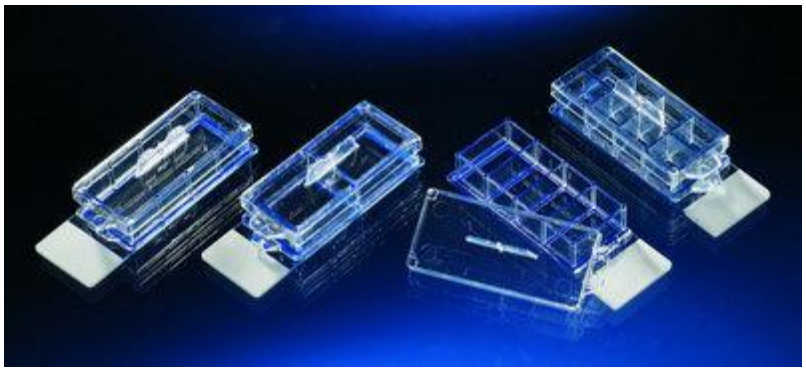


Figure 2.18: Nunc Lab-Tek^{TM} Chamber Slide^{TM} System. 8 well glass slides were used in this study for confocal imaging of RAW 264.7. Cells were cultured, stimulated and labelled directly on the slide. http://www.thermoscientific.com/en/product/nunc-lab-tek-ii-chamber-slide-system.html (21-01-2014)

2.12.1. Oregon Green-V and FITC-V conjugation

In order to be able to visualise V recombinant proteins within the cells, they were labelled with Oregon-Green or FITC. 1mg of V recombinant protein was buffer exchanged into 0.1M $NaHCO_3$, pH 8.3 using Pierce Slide-A-Lyser mini dialysis micro-tubes.

Following this, 5mg of Oregon Green (OG) or 20mg of FITC isomer was dissolved in 500µl of DMSO, creating a solution of OG (10mg/ml) or FITC (40mg/ml). 500µl of recombinant protein (1mg/ml) was mixed with 250 µl of OG or FITC. The tube was sealed with parafilm, wrapped in aluminium foil and left to incubate away from light at 120RPM and 37^{o}C for 3 hours.

In order to separate the conjugated protein from the unconjugated OG/FITC, a Sephadex PD-10 column (GE Healthcare) was used. Prior to separating the conjugated protein, the Sephadex column was equilibrated with X1 PBS. The conjugated protein was added to the Sephadex column and allowed to run through the column by the use of gravity alone. This results in the bigger conjugated OG-protein/FITC-protein separating itself from the smaller unconjugated OG/FITC and eluting from the column first. Therefore, the first coloured fraction that appeared from the column was OG-protein/FITC-protein, which was collected, wrapped in aluminium foil, protecting it from light, and then stored at 4ºC.

2.12.2. Seeding procedures for confocal microscopy

In this project, RAW 264.7 cells were grown on 8 well glass slides (Nunc Lab-Tek™ Chamber Slide™ System) and both effect and colocalisation of V antigen with different receptors (TLR4, NLRP12 and NLRP3), pathways (Caspase p-20, MyD88, IRF3 and IRF7) and organelles (nucleus (TO-PRO) endosomes (EEA1)) were investigated.

To investigate the effect of V antigen on TLR4 and MyD88, eight time points were used per stimulation: unstimulated (0 hour); 1 hour LPS, 1 hour V antigen; and 1 hour LPS followed by either 1, 2, 4 or 6 hour(s) of V antigen. The slides were divided up as shown in Figure 2.19 (left layout).

To investigate the colocalisation of V antigen with the different receptors, pathways and organelles RAW 264.7 cells were incubated with either OG or FITC labelled V antigen. Four time points were used per stimulation: 10 minutes, 30 minutes, 1 hour and 2 hours. The slides were divided up as shown in Figure 2.19 (right layout).

Effect of V antigen	
Unstimulated	1 hour LPS
1 hour V antigen	1 hour V antigen + 1 hour LPS
1 hour LPS + 1 hour V antigen	1 hour LPS + 2 hour V antigen
1 hour LPS + 4 hour V antigen	1 hour LPS + 6 hour V antigen

Colocalisation of V antigen	
10 minutes	10 minutes
30 minutes	30 minutes
1 hour	1 hour
2 hours	2 hours

Figure 2.19: Layout of Lab-Tek™ Chamber Slide™ System. Cells were grown to confluency and stimulated. After fixing, the cells were incubated with primary antibody, washed and incubated with the corresponding secondary antibody. TO-PRO-3 iodine (Molecular Probes Invitrogen) was used as a nuclear stain.

To prepare the slides, 400µl DMEM was added to each well. One flask of cells was trypsonised, 10,000 cells were added to each well, and the slides were incubated overnight, making sure that they did not reach 100% confluency before carrying on with the stimulations. Cells were counted using a hemocytometer (Section 2.3).

The medium was aspirated from each well, and the 150µl was then added to each well. The cells were then stimulated. All aspirations and additions to the wells were made from the same corner, to minimise damage to the cells.

At each time point, the cells were fixed with 300µl 4% PFA for 15 minutes, washed twice with 400µl X1 PBS, and left in 400µl X1 PBS until the whole slide was ready.

2.12.3. Labelling procedures for confocal microscopy

Once ready, the 400µl was aspirated off and 150µl of PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin was added to each well. 3µl primary antibody was added to each well (Section 2.1.). The slides were left incubated overnight at room temperature.

The following day the wells were washed two times with 400µl of PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin. 150µl of PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin was then added to each well. 5µl of the relevant secondary antibody was added to each well and incubated for 45 minutes in the dark at room temperature. The cells were then washed twice with 400µl PBS/0.02% BSA/0.02% NaN_3/0.02% Saponin.

2.12.3.1. Anti-fade treatment

The well chambers and gasket were removed from the slide and the cells were treated with SlowFade Gold Antifade Reagent (Molecular Probes, Invitrogen). One drop of antifade reagent was placed in each well, and left for ten minutes. This reagent suppresses photobleaching and preserves the signal of the fluorescently labelled secondary antibody. A coverslip was then placed over the wells and any air bubbles were gently pushed out from underneath them. Excess antifade reagent was removed, and the cover slip was sealed down using two thin layers of clear nail varnish.

Slides were then imaged using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue).

2.13. Animal experimentation

The testing on animals for scientific research can be traced back to the fourth century BCE in Greek writings. In the last few centuries animal testing has ranged from the demonstration of the germ theory by Louis Pasteur in the late nineteenth century, the discovery of classical conditioning by Ivan Pavlov, the exploration of space. Animal testing in the early twentieth century saw insulin isolated from dogs along with the development of modern anaesthetics, antibiotics and anticoagulants. More recently animal testing has enabled the development of vaccines and drugs to combat HIV. The ground braking cloning of Dolly the sheep in 1996 is the pinnacle of animal research which highlights the scientific advances that have been made, that will help humans and non-humans alike, which would have otherwise been impossible without animal testing. Although highly controversial, animal testing has undoubtedly improved and saved millions of lives throughout the world.

Due to the controversial nature of animal testing many rules, guidelines and regulations have been put into place to protect animal welfare. Britain was relatively early in comparison to the rest of the world to put into practice the Britain Cruelty to Animals Act introduced in 1876, which was the first act that was concerned specifically with animal testing. Later the Animals (Scientific Procedures) Act 1986 (ASPA) was introduced, which has been modified but still is the basis of the ASPA to this day.

2.13.1. Animal model selection

Animal models used in animal testing include both non-vertebrates and vertebrates. Non-vertebrates include the widely used *Drosophila melanogaster* and *Caenorhabditis elegans*. Vertebrates include animals such as mice, rats guinea pigs, cats, dogs, fish, amphibians, larger domestic species such as pigs, goats, equines and primates including new and old world monkeys.

In the selection of a model organism for a specific experiment a number of factors have to be considered. Preliminary *in vitro* data should be used to help select the model. Animal models can come in a number of varieties where they can be tailored for a specific experiment. Animals can be out-bred, in-bred, mutants or transgenic. Out-bred animals ("stocks") are from closed undefined genetic colonies. These have good fertility, large litter sizes, are easy to mate, have a good growth curve and are usually relatively cheap. Inbred mice ("strains") are compromised in these factors. However, their benefit is that they are ~99% homozygous as a result of ≥20 generations of brother sister inbreeding. Mutant animals are an out-bred stock with a mutant gene these are tricky to breed and maintain. Dominant and recessive mutations can have a number of effects such as infertility and high birth mortality. Transgenic mice have had foreign DNA permanently incorporated into their genome resulting in the knock out (KO) of genes, for example. These mice often require high levels of maintenance, such as germ free environments if their immune system is compromised for example.

In this study the out-bred CD-1 male mouse was selected as an animal model of sepsis. *Yersinia pestis* V antigen was tested for its ability to reduce inflammation and lethal shock in the murine model of sepsis, by protecting or potentially reversing the progression of lethal sepsis in mice.

2.13.1.1. The CD-1 mouse

In this study out-bred CD-1 male mice at 6-8 weeks were used as an animal model of sepsis as advised by Professor Mervyn Singer and Dr. Richard Hotchkiss who both have well established endotoxin CD-1 mouse models of sepsis. Mice were also selected for their good fertility, good growth curve, large litter size and the ease at which they can be bred. CD1 mice, which are an outbred stock, are most often used in research projects requiring vigorous, economically priced animals, but in which considerations of specific genotype are of lesser importance. In the current study we

wanted to assess the effect of V antigen on the outcome of sepsis, where the genetic genotype is of lesser importance. This would also be reflected in a human population of we were testing V antigen – there would have been variable genetic genotype – thus this model is ideal for testing this inhibitor.

Figure 2.20: The CD-1 mouse. In this study the CD-1 mouse was selected as an animal model of sepsis. They have good fertility, a good growth curve, a large litter size and are easy to breed. http://www.biolasco.com.tw/Biolasco%20website%20English/SPF%20ra12.jpg (17/02/2010).

LPS and V antigen were administered via intraperitoneal injection.

2.13.2. Intraperitoneal injection

Intraperitoneal (i.p) injection is the injection of a substance into the peritoneal cavity. This allows large amounts to be injected producing a massive and rapid systemic immune response. The procedure is relatively quick and easy which reduces any stress and damage that may be caused to the animal, reducing these factors increases result validity.

The mouse is restrained so that its head was facing away and downward with its abdomen exposed (Figure 2.21), this allows the intestines to be under less pressure to reduce chance of internal damage. The needle is inserted, at a 30˚ angle, close to the midline on the animals right side horizontally in-line with the knees to a depth of 5mm

(Figure 2.21). Injection to the right side places the needle at the sight of the small intestines; again reducing risk of internal damage for the needle is less likely to hit smaller structures.

Figure 2.21: Intraperitoneal (i.p.) injection to a mouse. The needle is inserted close to the midline on the animals right side horizontally in-line with the knees. http://www.procedureswithcare.org.uk/wp-content/uploads/2010/07/DSC_2824.jpg (17/02/2014).

2.13.3. Injected substance concentration determination

The concentrations of substances to be injected were determined with preliminary experiments. In order to determine whether V antigen protects against septic shock, a mouse model had to be developed. The septic shock mouse model was designed to have 100% mortality at 24 hours post LPS administration. V antigen was tested to find optimum concentration for protection against LPS endotoxin induced septic shock.

2.13.3.1. Determination of LPS concentration for sepsis model

Male CD-1 mice (6-8 weeks) were randomly grouped (5-10 mice per group) and injected by intraperitoneal (i.p.) injection with different concentrations (50, 60, 70, 80mg/Kg) of *Escherichia coli* 055 (*E. coli* 055) LPS (List Biological Laboratories) using

a sterile 1ml syringe (BD Plastipak™ (Becton Dickinson)) with a 25 GA1 0.5x25mm needle (BD Microlance™ 3 (Becton Dickinson)). The syringe was prepared with the correct volume of *E. coli* 055 LPS to be administered. The mouse was then restrained so that its head was facing away and downward with its abdomen exposed (Figure 2.21). The needle was inserted, at a 30˚ angle, close to the midline on the animals right side horizontally in-line with the knees to a depth of 5mm (Figure 2.21). The needle was withdrawn and the mouse returned to the cage.

Once the optimum concentration of LPS required to induce septic shock and death at 24 hours was determined, it was kept constant throughout the *in vivo* inhibition studies.

2.13.3.1.1. Induction of sepsis in the CD-1 mouse

Intraperitoneal (i.p.) injection of LPS (60mg/Kg) was administered to the CD-1 mouse (male 6-8 weeks) using a sterile 1ml syringe (BD Plastipak™ (Becton Dickinson)) with a 25 GA1 0.5x25mm needle (BD Microlance™ 3 (Becton Dickinson)). The syringe was prepared with the correct volume of LPS, relative to mouse weight, to be administered. Death commenced at 24 hours.

2.13.3.2. Determination of optimal V antigen concentration

In order to determine the optimum concentration of V antigen (Kindly supplied by Professor R. Titball of the Ministry of Defence, Porton Down) that was needed to protect mice from LPS-induced septic shock, mice were administered different concentrations (500µg/Kg and 1000µg/Kg) of V antigen by i.p. injection 1 hour after LPS administration. Blood (50µl) was collected at certain time points after V antigen administration, over a 72 hour period, from the tail vein of mice (250µl of blood in total). The level of TNF-α and IL-1β in the serum was determined using a cytokine bead array system (CBA) (Becton Dickinson).

2.13.3.2.1. *In vivo* testing of V antigen

Intraperitoneal (i.p.) injection of V antigen 500µg/Kg (Kindly supplied by Professor R. Titball of the Ministry of Defence, Porton Down)) was administered to the CD-1 mouse (male 6-8 weeks) using a sterile 1ml syringe (BD Plastipak™ (Becton Dickinson)) with a 25 GA1 0.5x25mm needle (BD Microlance™ 3 (Becton Dickinson)). The syringe was prepared with the correct volume of V antigen to be administered. The needle was withdrawn and the mouse returned to the cage.

2.13.4. Cytokine analysis of blood samples

Blood samples from mice were collected at different times before or after LPS administration and frozen until the cytokine assays were performed. The Becton Dickinson (Oxford, UK) Mouse Inflammation cytokine bead array (CBA) (Becton Dickinson) system was used in order to determine the level of TNF-α, IL-6, MCP-1 and IL12p70 according to the manufacturer's instructions (Section 2.8.2.2).

2.14. Statistical analysis

Data were evaluated by analysis of variance (ANOVA) and the Dunnett multiple-comparison test using the GraphPad Prism 5 program. Where appropriate (comparison of two groups only), two-tailed *t* tests were also performed. Statistical differences were considered significant at the level of $p<0.05$. Experiments were performed using triplicate samples and were performed twice or more to verify the results.

With regards to the animal model and based on experimental experience with this model over several years we have calculated that we need a minimum group size of 8 for statistical power. Using G*Power program freely available from the University of Dusseldorf website (http://www.psycho.uni-dusseldorf.de/abteilungen/aap/gpower3) (Faul et al., 2007) we calculate that this experiment had an actual power of over 90% (>0.9). If we assume that a similar level of variability will occur in any future

experiments and utilising the power value (>0.9) derived from our existing data we calculate that we shall need a minimum group size of 8 animals.

Chapter 3

Results

3.1. V antigen purification

In order to investigate the effect that V antigen might have on the innate immune response, recombinant versions of V antigen had to be produced. V antigen was cloned in *Escherichia coli* and kindly supplied to us by Professor Richard Titball of the Ministry of Defence, Porton Down. In order to purify the recombinant V, the *E. coli* strain had to be cultured in Luria broth and the protein isolated using affinity chromatography.

Prior to investigating the effect on the innate immune system, the purity of V antigen had to be confirmed as well as to eliminate any traces of endotoxin from the purified proteins.

3.1.1. Purification of V antigen

In order to produce recombinant V antigen, the recombinant LcrV plasmid (pVG110) with a glutathione S-transferase (GST) tag was used which was kindly provided by Prof. Richard Titball (Chemical and Biological Defence Establishment, Porton Down, Salisbury, UK). The plasmid was transformed into *E. coli* BL21 cells as previously described by Carr et al. (1999).

The cloning and expression of the gene was done by Prof. Titball and colleagues as described in Carr et al. 1999. Briefly, the *lcrV* gene (Genebank accession number AAA27641) was amplified by PCR from the chromosomal DNA of *Y. pestis* strain GB using 500pmol of primers (Cruachem, Strathclyde, UK) homologous to the 5' and 3' ends of the gene (the 5' primer GATCGAATTC^{+}GAGCCTACGAACAAAACCCA and the 3' primer GGATCGTCGACTTA*CATAATTACCTCGTGTCA), respectively and cloned into pGEX-6P-2 (GE Healthcare) to make the recombinant plasmid, pVG110.

E. coli BL21 cells containing the recombinant plasmid were cultured in Luria broth containing 100mg/ml ampicillin. Cultures were placed in a 37ºC rotary incubator at

120RPM until an A_{600} of 0.3 was achieved; cultures were then induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 6 hours and harvested by centrifugation at 7000RPM for 30 minutes (Section 2.5.2).

Harvested cells were resuspended in PBS and lysed by repeated freeze-thaw cycles. Crude cell extract was clarified by centrifugation at 2500RPM for 30 minutes and the supernatant added to glutathione Sepharose columns, previously equilibrated with PBS. Unbound material was washed off with PBS. Cleavage buffer (10ml) (50mM Tris/150mM NaCl/1mM EDTA/1mM DTT/0.01%) with PreScission™ Protease (150µl) was added to the column and incubated at room temperature for 3 hours. The column was then washed with more cleavage buffer (20ml) and the elution was collected (Section 2.5.5).

The elution was concentrated and subsequently passed through a Profos EndoTrap® blue 10 to ensure there was no LPS in the final solution (Section 2.5.7.1). The HyCult LAL Chromogenic Endotoxin Quantitation Kit was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* Amebocyte Lysate (LAL) assay, which was found to be none (Section 2.5.8).

The preparations of purified rV were analysed by SDS-PAGE followed by Coomassie blue staining in order to verify its purity (Section 2.9.4). For Western blotting, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and detected using a V antigen specific monoclonal antibody (mouse anti-LcrV (Abcam)) at 1:1000 dilution followed by a goat anti-mouse IgG horse-radish peroxidase conjugate at 1:2000 dilution.

The molecular weight of the protein was determined by SDS-PAGE and was found to be 38 kDa, which is within the predicted molecular weight range of native V antigen (Figure 3.1). Although some lower molecular weight proteins were observed after

Coomassie staining (which could be V antigen fragments), the major band observed was the 37kDa V antigen.

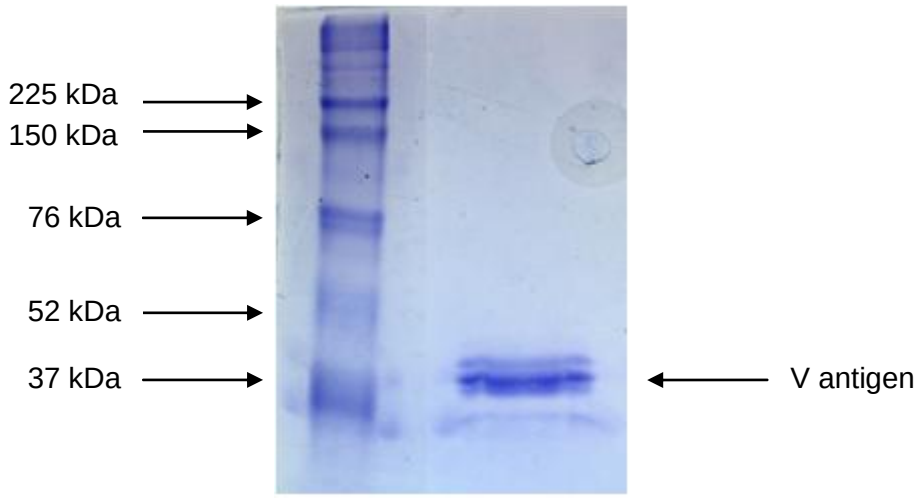


Figure 3.1: Coomassie blue stain for detection of purity of V antigen. 5µg of V antigen was analysed by SDS-PAGE and subsequently stained with Coomassie blue stain. Molecular weight markers were used to determine the correct position of the molecular weight of the bands indication presence of the desired protein.

A strong band appeared around 37kDa, which suggests that there were no other proteins present except V antigen. In order to verify the identity of the 37kDa band observed with the Coomassie blue stain, a western blot was performed using an anti-V antigen specific antibody (Figure 3.2). It was shown that the band identified in the Coomassie blue stained gel, was indeed V antigen. Some minor band of lower and higher molecular weight appeared on the western blot and must represent fragments and aggregates of V antigen since they are recognised by the mAb specific for V antigen.

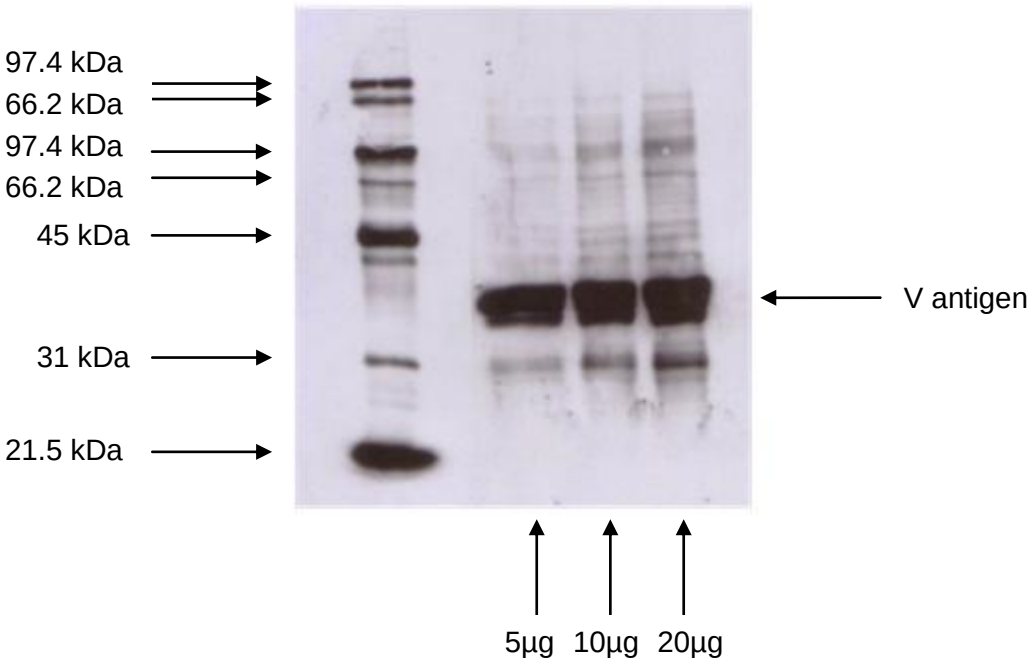


Figure 3.2: Western blots for detection of V antigen at different concentrations. Molecular weight markers were used to determine the correct position of the molecular weight of the bands indicating presence of the desired protein. The loading volumes in the wells were 5µg, 10µg and 20µg, respectively, of the purified solution.

3.1.2. Conclusions

In order to investigate the effects of *Y. pestis* V antigen on human monocytes/macrophages, recombinant proteins had to be produced. Prof. Richard Titball kindly provided us with the recombinant LcrV plasmid (pVG110) with a gluthione S-transferase (GST) tag. The plasmid was transformed into *E. coli* BL21 cells as previously described by Carr et al. (1999).

The LcrV protein was encoded with a glutathione S-transferase (GST) tag, thus it was straight forward to isolate the protein from the crude *E. coli* cell extract. LcrV recombinant proteins were isolated using glutathione Sepharose columns which have affinity for the GST tag, and subsequently eluted using PreScission™ Proteases. The eluted proteins were buffer-exchanged, concentrated and all traces of endotoxin

removed by passing the protein through Profos EndoTrap® blue 10 columns. In order to verify that all the endotoxin was removed, the *Limulus* Amebocyte Lysate (LAL) assay was used, which found no traces of endotoxin.

The preparations of purified recombinant V antigen (rV) were analysed by SDS-PAGE followed by Coomassie blue staining. It was shown that the molecular weight of the protein was 37kDa, which was the predicted molecular weight of native V antigen. Some lower molecular weight proteins were observed after Coomassie staining (which could be V antigen fragments), the major band observed was the 37kDa V antigen. Western blotting also verified that the main protein band at 37kDa was V antigen, whereas smaller fragments also seemed to be present.

The initial stage of this study was to generate and characterise recombinant V antigen. In order to investigate the effects of V antigen on the innate immune responses, recombinant protein that was pure and endotoxin free had to be produced. Once this was achieved the effects of this protein on the innate immune response was investigated.

3.2. *Yersinia pestis* V antigen induced immunomodulatory effects in THP-1 cells

Regulation of the innate immune system is important to keep the body in a healthy state. A loss of regulation or excessive stimulation of innate immunity can lead to a dangerous and often fatal symptoms. Septic shock is the purest example of this deregulation, where an overreaction of the host innate immune system lead to harmful symptoms. The search to pinpoint molecules that are able to modulate the innate immune responses is decisive in the successful development of a therapeutic intervention for sepsis.

Yersinia pestis, the causative agent of the three main plague pandemics has been responsible for over 200 million deaths over several millennia. The bacterium has been so successful because it encodes several virulence factors, one of which is LcrV (V antigen). *Y. pestis* V antigen is a multifunctional protein that has been shown to exhibit immunomodulatory properties and essential in translocating other virulence factors into eukaryotic cells.

It is the hypothesis of this study that this multifunctional protein modulates the innate immune responses to the bacterial component lipopolysaccharide (LPS), ultimately reversing the detrimental effects of sepsis associated inflammatory response. This study utilised monocytic (THP-1) (Section 2.2.2) cells to elucidate whether LcrV affects pattern recognition receptors (PRRs) and the subsequent inflammatory cytokine release. Firstly we investigated the effect of LPS on PRR expression and its capability of initiating an innate immune response in THP-1 cells.

3.2.1. Effect of LPS on TLR, CD14, NOD and NLRP expression in THP-1 cells.

In order to determine which PRRs are involved in the recognition of LPS, THP-1 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of *E. coli* LPS (Section 2.6). All stimulations were carried out in 25cm^2 flasks. The receptors (TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, NOD1, NOD2, NLRP1, NLRP3 and NLRP12) were labelled and their expression levels detected using indirect immunofluorescence (Section 2.7.2) and analysed by flow cytometry (Section 2.8). In addition the expression levels of CD14 were also determined as it is a co-receptor of TLR4. Unstimulated cells were used as a control of THP-1 receptor expression levels prior to stimulation.

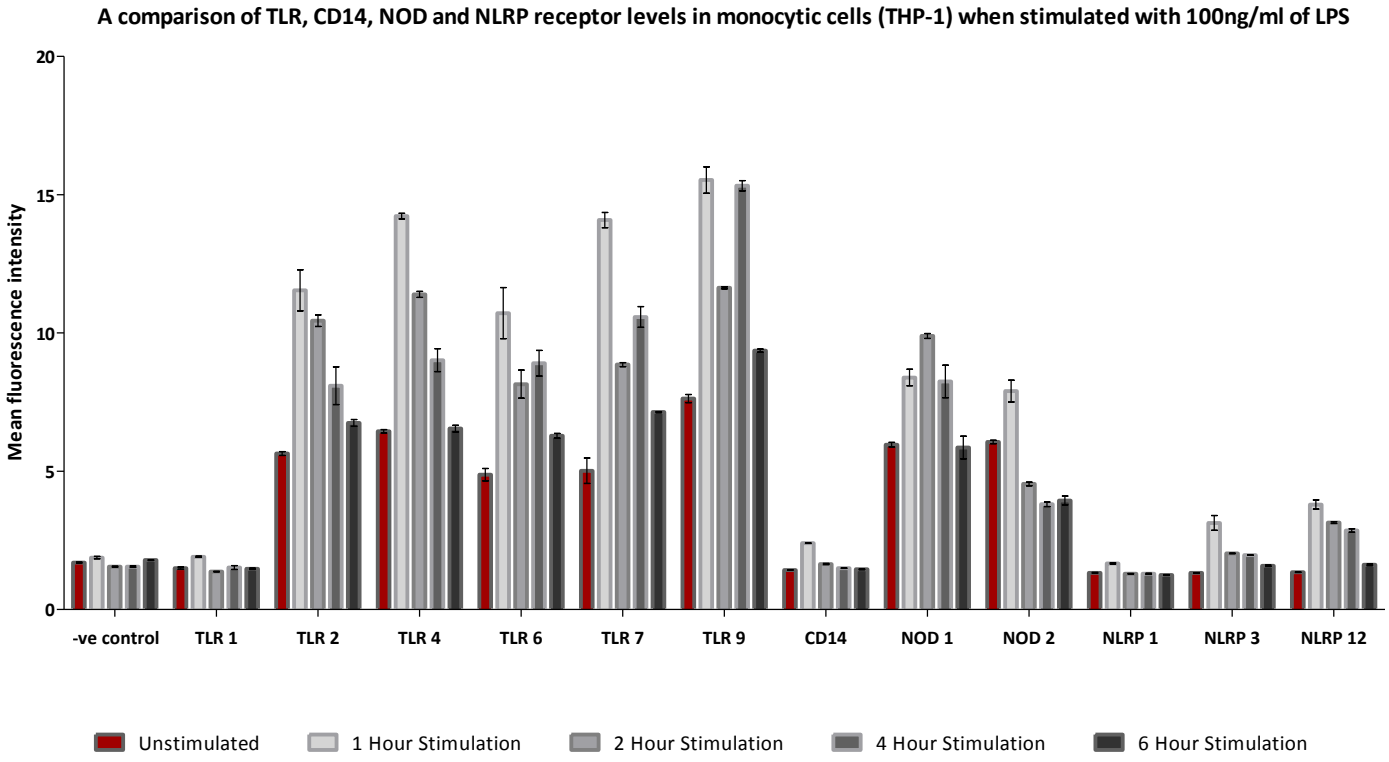


Figure 3.3: PRR expression levels on THP-1 cells in response to 100ng/ml of LPS. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on THP-1 cells stimulated with 100ng/ml of LPS for 1, 2, 4 and 6 hours. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM}

(Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

LPS caused up-regulation of TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12 expressed on THP-1 cells (Figure 3.3). There was no up-regulation of TLR1 and NLRP1 observed. In response to LPS, the receptors TLR2, TLR4 and co-receptor CD14 expression decayed over time with a maximum at 1 hour, possibly due to internalisation of the receptors. Likewise, NOD2, NLRP3 and NLRP12 expression decayed over time with, a maximum at 1 hour. With regards to TLR6, TLR7 and TLR9 expression, LPS induces up-regulation at 1 hour, the highest expression, followed by a downregulation at the 2 hour mark and a second increase of expression at 4 hours. A downregulation reoccurred by the 6 hours time-point, whose expression level was the lowest. Furthermore, after LPS stimulation, there is a marked increase in NOD1 expression after the first two hours, which tails off after 4 and 6 hours of stimulation.

As expected, lipopolysaccharide has a broad effect on the expression levels of THP-1 cells, up-regulating most pattern recognition receptors, either directly or indirectly by the secretion of cytokines.

3.2.2. Effect of LcrV on TLR, CD14, NOD and NLRP expression in THP-1 cells.

In order to test the effect of *Yersinia pestis* V antigen has on the expression of different PRRs, THP-1 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. All stimulations were carried out in $25cm^2$ flasks. The expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and

NLRP12 were labelled and detected using indirect immunofluorescence (Section 2.7.2) and analysed by flow cytometry (Section 2.8).

Initially, THP-1 cells were stimulated with 50µg/ml of V antigen (Figure 3.4). The expression levels of TLR2, TLR4, TLR6, TLR7, NOD1 and NLRP12 were inferior in comparison to THP-1 cells stimulated for 1 hour with LPS. In contrast, the expression levels of TLR9, CD14, NOD2, NLRP1 and NLRP3 were greater in response to V antigen. In particular, NOD2 showed a noticeable increase when cells were stimulated with V antigen in comparison to stimulation with LPS for 1 hour. TLR1 did not manifest an evident down or up-regulation.

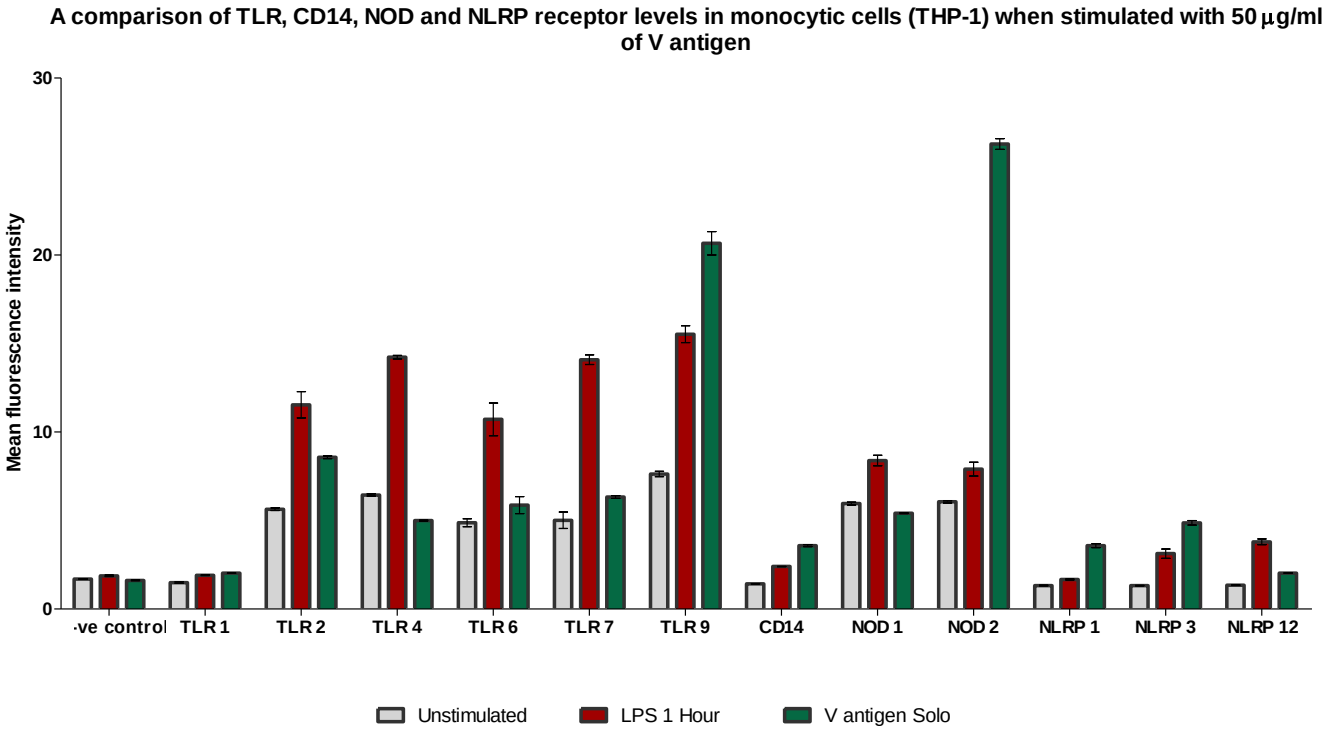


Figure 3.4: PRR expression levels on THP-1 cells stimulated for 1 hour with 50µg/ml of V antigen. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on THP-1 cells stimulated for 1 hour with 50µg/ml of V antigen. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Subsequently, THP-1 cells were pre-incubated with 50µg/ml of V antigen followed by stimulation with 100ng/ml of LPS (Figure 3.5). This pre-incubation caused up-regulation of TLR2, TLR6 and TLR7. In addition, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 expression levels also increased with pre-incubation with V antigen followed by a LPS stimulation. Furthermore, the TLR4 co-receptor CD14 also showed an increase in expression. After the pre-incubation with V and stimulation with LPS, TLR1 and TLR4 showed no marked change in expression, whereas TLR9 showed a downregulation.

A comparison of TLR, CD14, NOD and NLRP receptor levels in monocytic cells (THP-1) when pre-incubated for 1 hour with 50µg/ml of V Antigen and stimulated for 1 hour with 100ng/ml of LPS

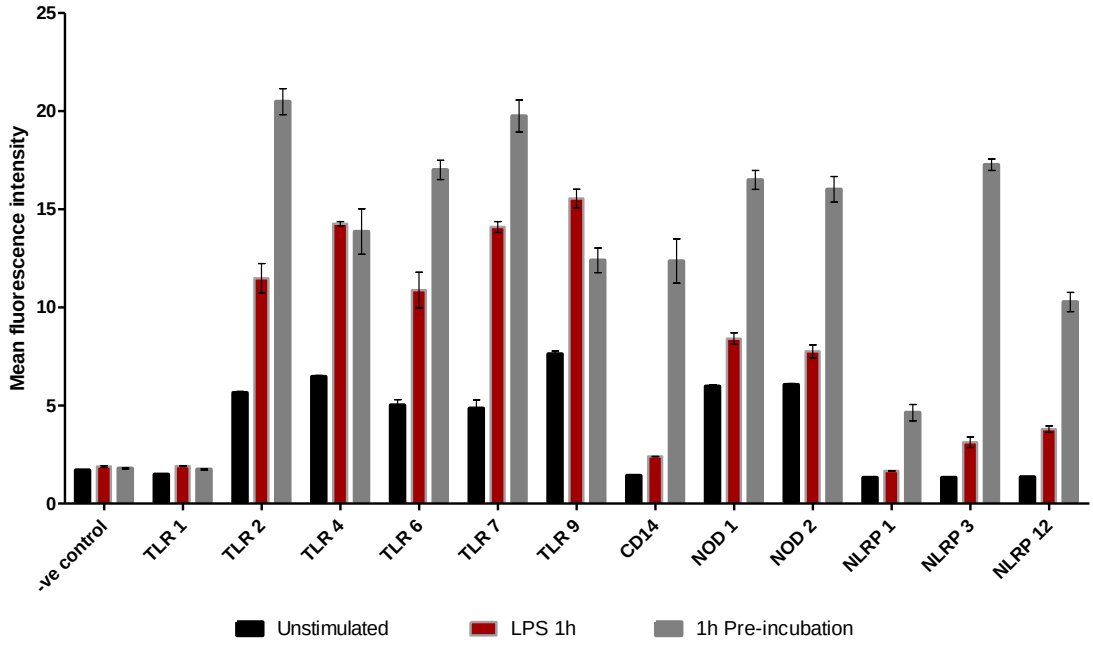


Figure 3.5: PRR expression levels on THP-1 cells pre-incubated for 1 hour with 50µg/ml of V antigen and stimulated for 1 hour with 100ng/ml of LPS. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on THP-1 cells pre-incubated for 1 hour with 50µg/ml of V antigen and stimulated for 1 hour with 100ng/ml of LPS. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

The following step was to examine what effect post-incubating THP-1 cells with V antigen, after stimulating them with LPS, has on the expression of the different PRRs (Figure 3.6).

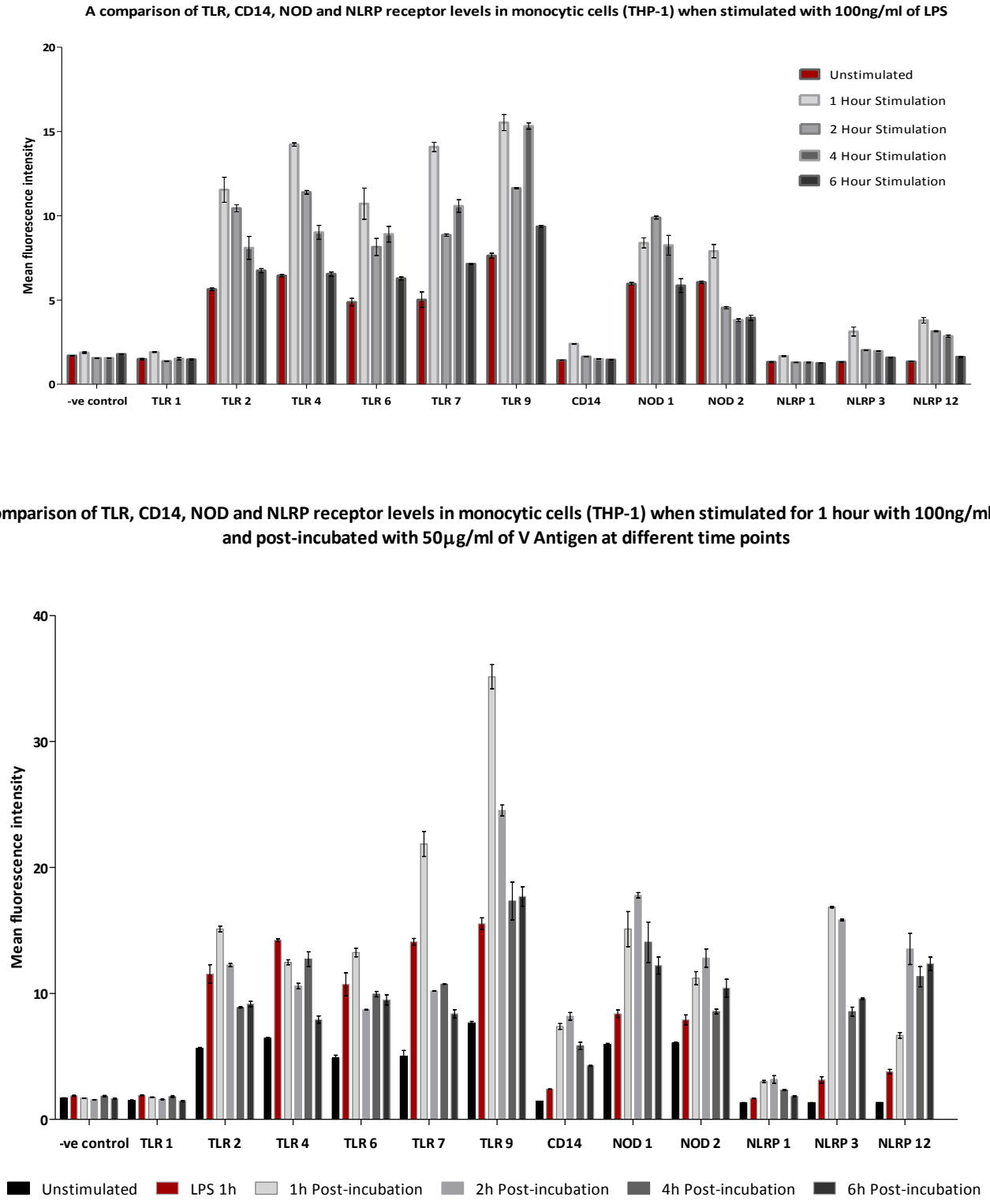


Figure 3.6: PRR expression levels on THP-1 cells stimulated for 1 hour with 100ng/ml of LPS and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on THP-1 cells stimulated for 1 hour with 100ng/ml of LPS and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

TLR4 expression levels seem to downregulate in response to post-incubation with V antigen, with the lowest expression registered at 6 hour post-incubation. With regards to the receptors TLR6 and TLR7, they seem to have a similar trend of an initial increase of expression by the first hour of post-incubation followed by a drop in expression for the remaining time-points. The expression level of TLR2 in response to post-incubation with V antigen initially increased, with a maximum at 1 hour post-incubation, which tails off for the remaining post-incubation time-points, noting that at the 4 and 6 hour post-incubation mark the expression levels are lower compared to the expression of TLR2 in response to LPS alone.

At 1 hour post-incubation TLR9 expression level doubled, compared to cells stimulated solely with LPS. The following post-incubation time-points show a marked decrease in expression, where at 4 and 6 hours the expression level decreased to approximately the level observed in cells stimulated only with LPS. NLRP3 showed a similar trend to TLR9, where an initial surge in expression level was observed, followed by a decline in expression. It differed from the results obtained from TLR9 where at 4 and 6 hours the expression level of NLRP3 was higher compared to the one observed in cells stimulated with just with LPS.

The TLR4 co-receptor CD14 and NOD1 expression levels increase after the first two post-incubation time-points, peaking at 2 hours post-incubation, which tails off after 4 and 6 hours of post-incubation with V antigen. NLRP1 seems to have a similar response to post-incubation with V antigen as CD14 and NOD1, although a weak expression increase was observed.

Finally, the expression levels of NOD2 and NLRP12 in response to post-incubation with V antigen induced an up-regulation for the first two hours post-incubation, the highest expression being at the second post-incubation mark, followed by a downregulation at

the 4 hour and a second increase of expression at 6 hours post-incubation with V antigen.

3.2.3. Inflammatory immune responses triggered by *Yersinia pestis* V antigen

As previously mentioned, one of the consequences of activation of TLRs is the expression of genes via NF-κB which, normally, is sequestered by unphosphorylated IκB. One of the most shared signalling pathways from TLR activation leads to the phosphorylation of IκB and consequently the release of NF-κB, which in turn lead to the production of inflammatory cytokines. Furthermore, also mentioned above, inflammasome sensor molecules, such as NODs and NLRPs, can activate caspase-1 where its main role is to convert pro-IL-1β and pro-IL-18 into their bioactive cytokine IL-1β and IL-18, respectively.

3.2.3.1. NF-κB and caspase-1 activation in THP-1 cells in response to V antigen

The protein complex NF-κB is the transcription factor that is involved in the cellular response to stress and, of particular interest to this work, presence of cytokines. The presence of phospho-IκB in the cell lysate indicates the transcription of pro-inflammatory cytokines. Similarly, the presence of caspase-1 is an indicative of inflammasome activation, which also leads to a secretion in inflammatory cytokines. Western blotting was utilised in order to determine the presence of both phospho-IκBα and caspase-1 p-10.

Since V antigen was shown to be able to modulate the expression of different PRRs, experiments were performed in order to test the hypothesis that V antigen is able to modulate NF-κB and caspase-1 activation in response to LPS.

3.2.3.1.1. Presence of phospho-IκBα and caspase-1 p10 in THP-1 cells in response to LPS

Initially, in order to determine whether LPS can lead to NF-κB and caspase-1 activation, THP-1 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. The supernatant was collected, at the respective time-points, frozen and stored for later cytokine assays. The cells were lysed and the samples were separated by size via SDS-PAGE (Section 2.9) and transferred onto a membrane (Section 2.10.1), which was probed for phospho-IκBα or caspase-1 p-10, with their respective primary antibody, followed by the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP). Membranes were imaged via enhanced chemiluminescence (Section 2.11). It was shown that LPS triggers NF-κB (Figure 3.11) and caspase-1 (Figure 3.12).

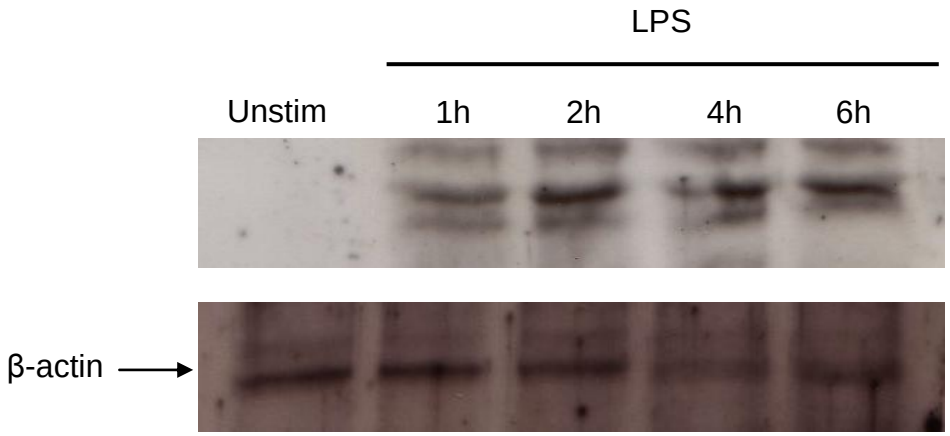


Figure 3.11: Western blot of phospho-IκBα from lysates of unstimulated THP-1 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

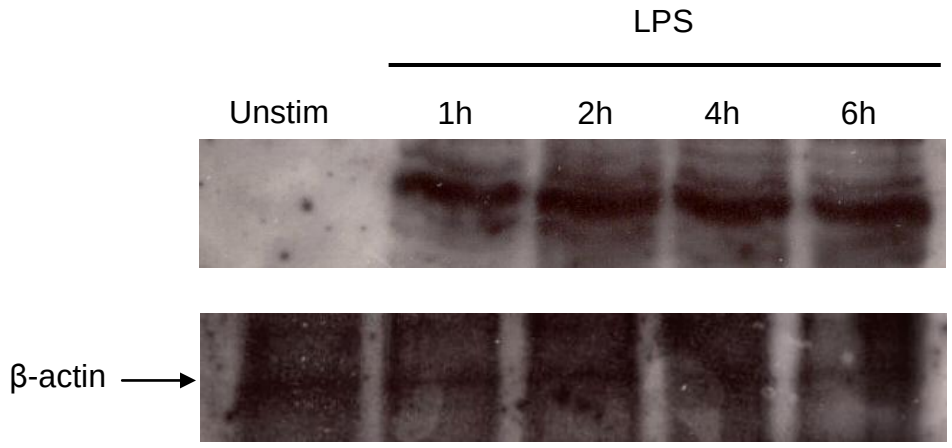


Figure 3.12: Western blot of caspase-1 p10 from lysates of unstimulated THP-1 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

3.2.3.1.2. Presence of phospho-IκBα and caspase-1 p10 in THP-1 cells in response to LPS pre- and post-incubated with V antigen

Once it was established that LPS is able to trigger NF-κB signalling cascade and caspase-1 activation in THP-1 cells, whether V antigen was able to modulate this response was investigated. In order to do so, THP-1 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50μg/ml of V antigen. The cells were lysed and the samples were separated by size via SDS-PAGE (Section 2.9) and transferred onto a membrane (Section 2.10.1), which was probed for phospho-IκBα or caspase p-10, with their respective primary antibody, followed by the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP). Membranes were imaged via enhanced chemiluminescence (Section 2.11).

The results obtained from pre-incubating V antigen show that there is still some activation of NF-κB in response to LPS, however it appears to be slightly inhibited. On the other hand, the results obtained from cells stimulated with LPS and subsequently post-incubated with V antigen show that the activation of NF-κB is inhibited (Figure 3.13).

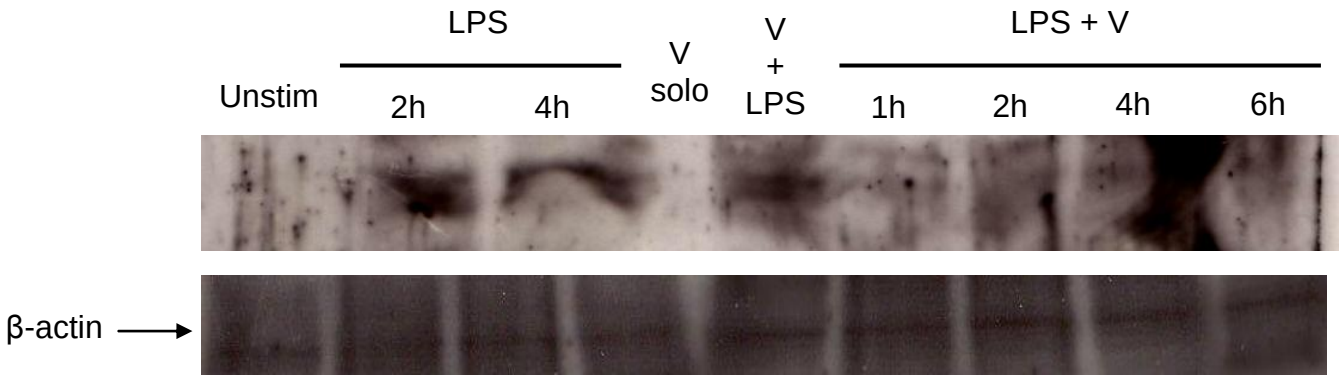


Figure 3.13: Western blot of phospho-IκBα from lysates of unstimulated THP-1 cells; stimulated for 2 and 4 hours with 100ng/ml LPS; stimulated for 1 hour with 50µg/ml of V antigen; pre-incubated for 1 hour with 50µg/ml of V antigen and subsequently stimulated for 1 hour with 100ng/ml LPS; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

Concerning caspase-1 activation, the results show that both pre- and post-incubations with V antigen result in a reduction of caspase-1. This reduction seems to gradually occur through the first three post-incubation time-points, with a maximum at the 4 hour post-incubation mark, and at the 6 hour mark it seems to re-increase caspase-1 activation (Figure 3.14).

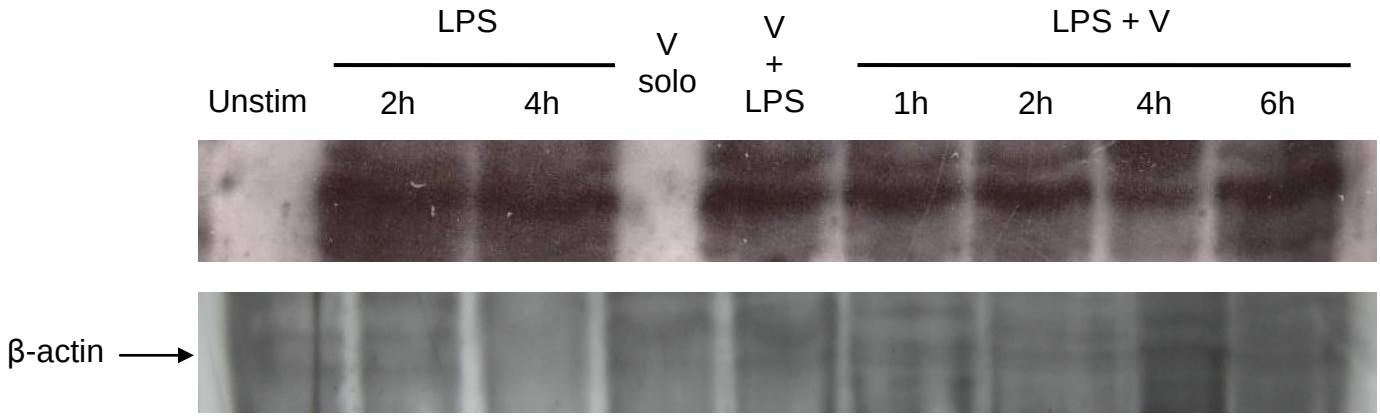


Figure 3.14: Western blot of caspase-1 p10 from lysates of unstimulated THP-1 cells; stimulated for 2 and 4 hours with 100ng/ml LPS; stimulated for 1 hour with 50µg/ml of V antigen; pre-incubated for 1 hour with 50µg/ml of V antigen and subsequently stimulated for 1 hour with 100ng/ml LPS; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

3.2.3.2. Cytokine secretion in THP-1 cells in response to V antigen

Given that V antigen was found to modulate the expression of PRRs and the activation of both NF-κB and caspase-1 activation, the next step in this study was to examine the release of cytokines, the inflammatory mediators, caused by LPS and whether or not V antigen is able to modulate their secretion. Out of the inflammatory cytokines analysed (Interlukin-8 (IL-8), Interlukin-1β (IL-1β), Interlukin-6 (IL-6), Interlukin-10 (IL-10), Tumor Necrosis Factor (TNF) and Interlukin-12p70 (IL-12p70)), only the release of IL-1β, IL-6, IL-10 and TNF were significantly increased.

THP-1 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with different concentrations (50µg/ml and 100µg/ml) of V antigen. Additionally, THP-1 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. All stimulations were carried out in 25cm^2 flasks. The supernatants were collected post

stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

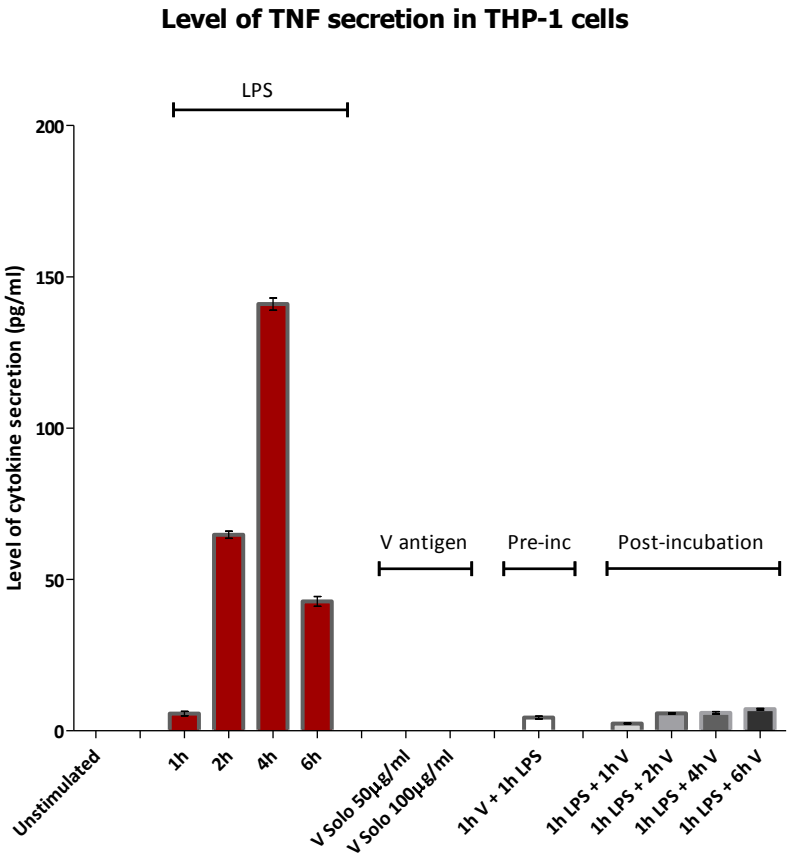


Figure 3.15: Secretion of TNF in THP-1 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. THP-1 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml or 100µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As expected, it was shown that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then decreased considerably at the 6 hour mark. It was also shown that V antigen on its own did not stimulate TNF secretion at any

concentration tested. TNF secretion was significantly down-regulated in response to pre- and post-incubation with V antigen (Figure 3.15).

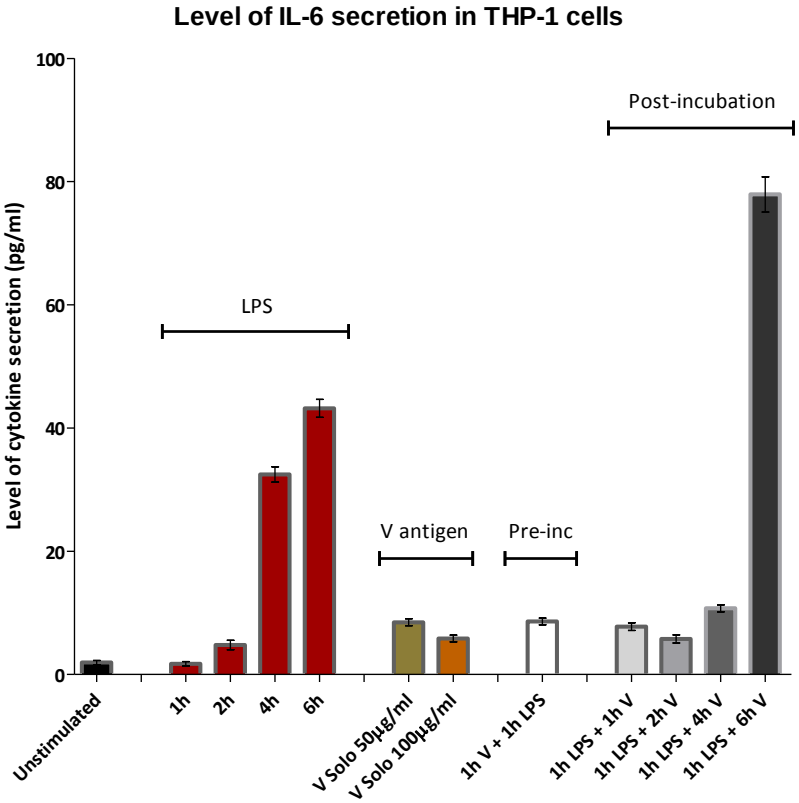


Figure 3.16: Secretion of IL-6 in THP-1 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. THP-1 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml or 100µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

When THP-1 cells were stimulated with LPS they incrementally secreted IL-6, with a maximum at 6 hour stimulation. Both 50 and 100µg/ml of V antigen by itself did also stimulate IL-6 production, although not to the same extent as the 4 and 6 hour LPS

stimulation. The same can be said to THP-1 cells pre-incubated with V antigen. The first 3 post-incubation time-points with V antigen, the levels of IL-6 were much lower than the 4 and 6 hour LPS stimulation, although at the 6 hour post-incubation mark a acute increase in IL-6 secretion occurred (Figure 3.16).

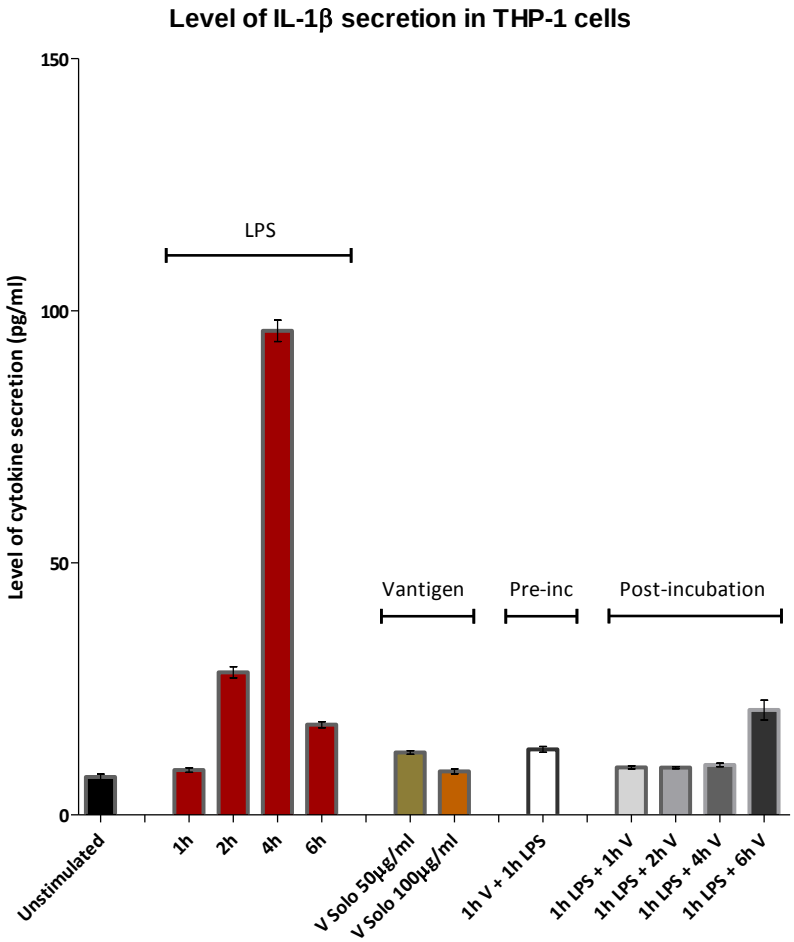


Figure 3.17: Secretion of IL-1β in THP-1 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. THP-1 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml or 100µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

The secretion of IL-1β in THP-1 gradually increased in response to LPS, with a maximum at the 4 hour stimulation, followed by a downregulation at the 6 hour mark. V

antigen on its own stimulated IL-1β secretion. Both pre- and post-incubation with V antigen resulted in a general downregulation of the level of IL-1β. It seems that at the 6 hour post-incubation time-point the level of IL-1β increases slightly compared to the previous time-points, although it still is a lower level than when cells are stimulated with LPS (Figure 3.17).

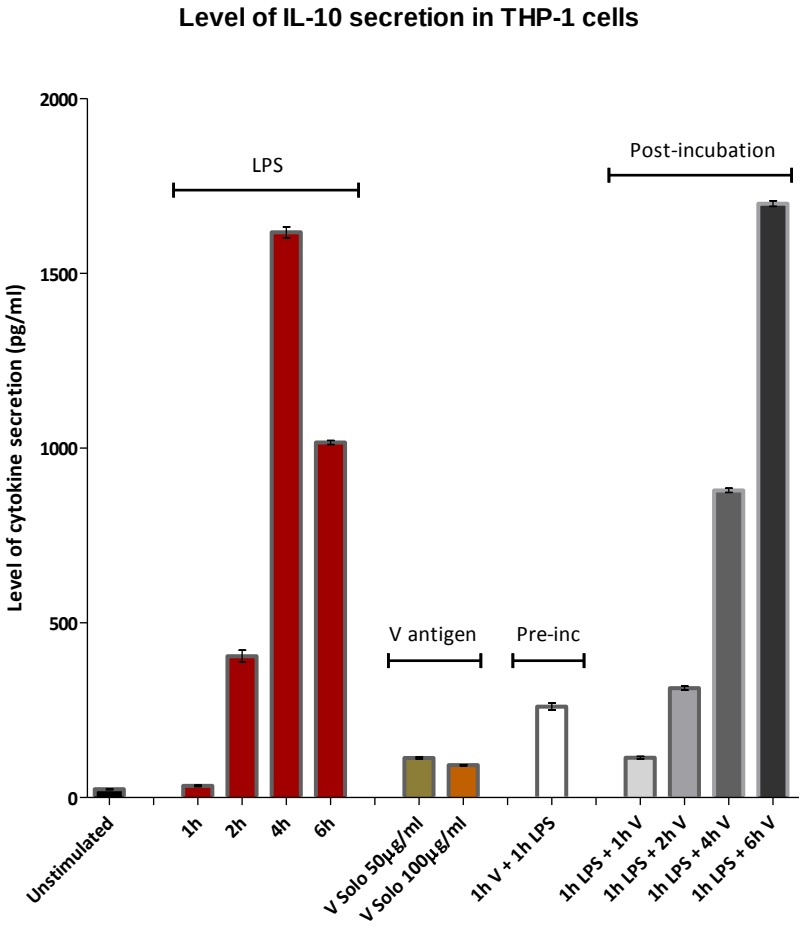


Figure 3.18: Secretion of IL-10 in THP-1 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. THP-1 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml or 100µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

When THP-1 cells were stimulated with LPS they gradually secreted IL-10 in response to LPS, with a maximum at the 4 hour stimulation, followed by a downregulation at the 6 hour mark. THP-1 cells stimulated with either 50 or 100µg/ml of V antigen by itself resulted in a slight IL-10 production. Pre-incubating THP-1 cells with V antigen resulted in a production of IL-10. The same cannot be said to THP-1 cells post-incubated with V antigen, as the production of IL-10 seemed to increase progressively through the time-points with V antigen, where the secreted levels reached a maximum at the 6 hour post-incubation mark.

3.2.4. Conclusion

Yersinia pestis V antigen (LcrV) is a multifunctional protein and has been shown to exhibit immunomodulatory features and is essential in translocating other virulence factors into eukaryotic cells. One of the aims of this study was to investigate the effects of V antigen on innate immune responses.

Initially, THP-1 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS and the expression levels of different PRRs were investigated. TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12 were all affected by the stimulation with LPS, either by direct interaction (CD14, TLR4) or by indirect interaction via the secretion of cytokines (all the other PRRs). In order to determine whether V antigen could immunomodulate innate immune responses, THP-1 cells were stimulated for 1 hour with 50µg/ml of recombinant V antigen and the expression levels of the different PRRs were again examined. This showed a lesser expression level of TLR2, TLR4, TLR6, TLR7, NOD1 and NLRP12, and, on the other hand, the expression levels of TLR9, CD14, NOD2, NLRP1 and NLRP3 was greater in response to V antigen, in comparison with cells stimulated with LPS. This demonstrated that V antigen can affect the expression levels of PRRs.

Subsequently, THP-1 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. Pre-incubating THP-1 cells with V antigen caused up-regulation of TLR2, TLR6, TLR7, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12. On the other hand, after the pre-incubation with V and stimulation with LPS, TLR1 and TLR4 showed no marked change in expression, whereas TLR9 showed a downregulation. Post-incubating THP-1 cells with V antigen triggered downregulation of TLR2, TLR4, TLR6, TLR7. On the contrary, CD14, NOD1, NOD2, NLRP3 and NLRP12 expression levels seem to up-regulate. TLR9 showed an initial up-regulation, followed by a considerable down-regulation. There was no marked change in expression levels of TLR1 and NLRP1.

Since our results showed that V antigen was able to modulate the expression of different PRRs in THP-1 cells, the next step was to test the hypothesis that V antigen is able to modulate NF-κB and caspase-1 activation, and as a consequence the release of inflammatory cytokines. Initially, we tested whether in out model cell system, the THP-1 cells could activate NF-κB and caspase-1, when stimulated with LPS. Our results showed that THP-1 cells do activate both NF-κB and caspase-1. Once it was established that LPS was able to trigger NF-κB signalling cascade and caspase-1, whether V antigen was able to modulate this response was investigated. Our results revealed that in THP-1 cells V antigen inhibited NF-κB activation.

With reference to inflammatory cytokine release, V antigen seemed to inhibit completely TNF secretion. Similarly, IL-1β secretion levels seem to be inhibited in response to V antigen, although a very slight increase in secretion level was observed at the 6 hour post-incubation mark in THP-1 cells. In addition, cells IL-6 secretion seems to be inhibited in the first 3 post-incubation time-points and an acute increase occurred at the 6 post-incubation hour, whereas IL-10 cytokine production seemed to

be up-regulated in response to V antigen. It increased progressively through the time-points reaching a maximum at the 6 hour post-incubation mark.

Taken together our data suggests that V antigen is able to modulate not only the expression levels of different PRRs, but also inhibit the signalling pathways that lead to the secretion of pro-inflammatory cytokines.

3.3. *Yersinia pestis* V antigen induced immunomodulatory effects in RAW 264.7 cells

In this study we utilised human THP-1 cells and mouse RAW 264.7 cells before moving to our *in vivo* model. We utilised RAW 264.7 cells to further elucidate whether LcrV affects pattern recognition receptors (PRRs) and its capability of initiating an innate immune response and as a natural *in vitro* step before using our sepsis CD1 mouse model.

3.3.1. Effect of LPS on TLR, CD14, NOD and NLRP expression in RAW 264.7 cells.

In order to determine which PRRs are involved in the recognition of LPS, RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml (Section 2.6). All stimulations were carried out in 25cm^2 flasks. The expression levels of the receptors (TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, NOD1, NOD2, NLRP1, NLRP3 and NLRP12) were labelled and detected using indirect immunofluorescence (Section 2.7.2) and analysed by flow cytometry (Section 2.8). In addition the expression levels of CD14 were also determined as it is a co-receptor of TLR4. Unstimulated cells were also used as a control of RAW 264.7 receptor expression levels prior to stimulation.

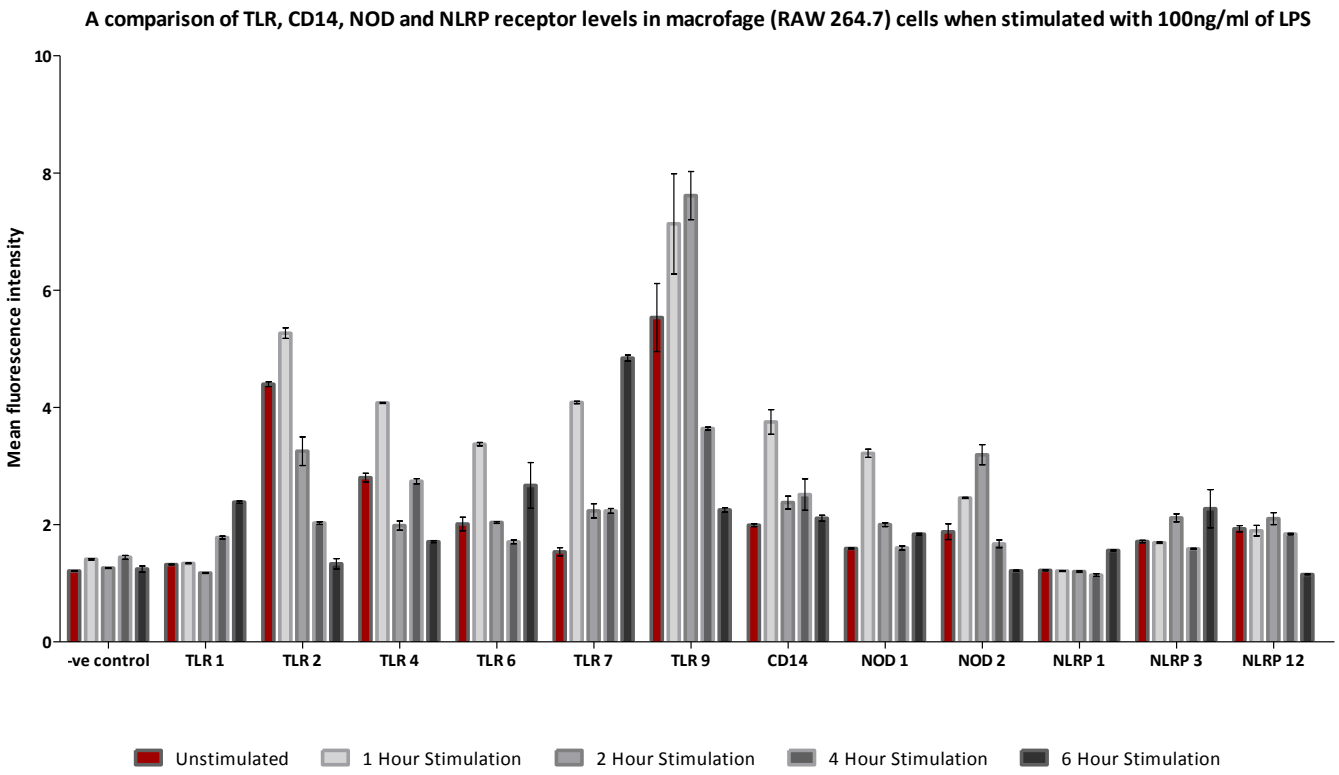


Figure 3.7: PRR expression levels on RAW 264.7 cells in response to 100ng/ml of LPS. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on RAW 264.7 cells stimulated with 100ng/ml of LPS for 1, 2, 4 and 6 hours. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

In the case of TLRs and TLR-associated molecules, LPS caused up-regulation of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14 expressed on RAW 264.7 cells (Figure 3.3). For all TLRs, LPS stimulation induced up-regulation of the receptors at 1 hour, the highest expression, followed by a downregulation at the 2 hour, whereas the PRRs recovered by 4 hours. With regards to TLR9 and NOD2 expression, LPS induces up-regulation at the first 2 hours, the second hour being the highest expression, followed by a downregulation at the 4 and 6 hour mark. An up-regulation was observed in the

TLR4 co-receptor CD14 and NOD1 in the first hour of stimulation with LPS. This was followed by a general decrease of expression for the remaining three time-points.

In the case of NLRs, NLRP1, NLRP3 and NLRP12 remained virtually unchanged in response to LPS, whereas NOD1, NOD2 were found to be up-regulated within 1 hour and 2 hours of LPS stimulation, respectively, followed by a downregulation at 2 hours and 4 hours, respectively.

As expected, lipopolysaccharide has a broad effect on the expression levels of RAW 264.7 cells, up-regulating most pattern recognition receptors.

3.3.2. Effect of LcrV on TLR, CD14, NOD and NLRP expression in RAW 264.7 cells.

In order to test the effect of *Yersinia pestis* V antigen has on the expression of different PRRs, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. All stimulations were carried out in 25cm^2 flasks. The expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 were labelled and detected using indirect immunofluorescence (Section 2.7.2) and analysed by flow cytometry (Section 2.8).

Firstly, RAW 264.7 cells were stimulated with 50µg/ml of V antigen (Figure 3.8). V antigen caused a general lowering of PRR expression. In response to V antigen TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12 expression levels lowered in comparison to RAW 264.7 cells stimulated for 1 hour with LPS. NLRP1 shows a slight decrease in its expression.

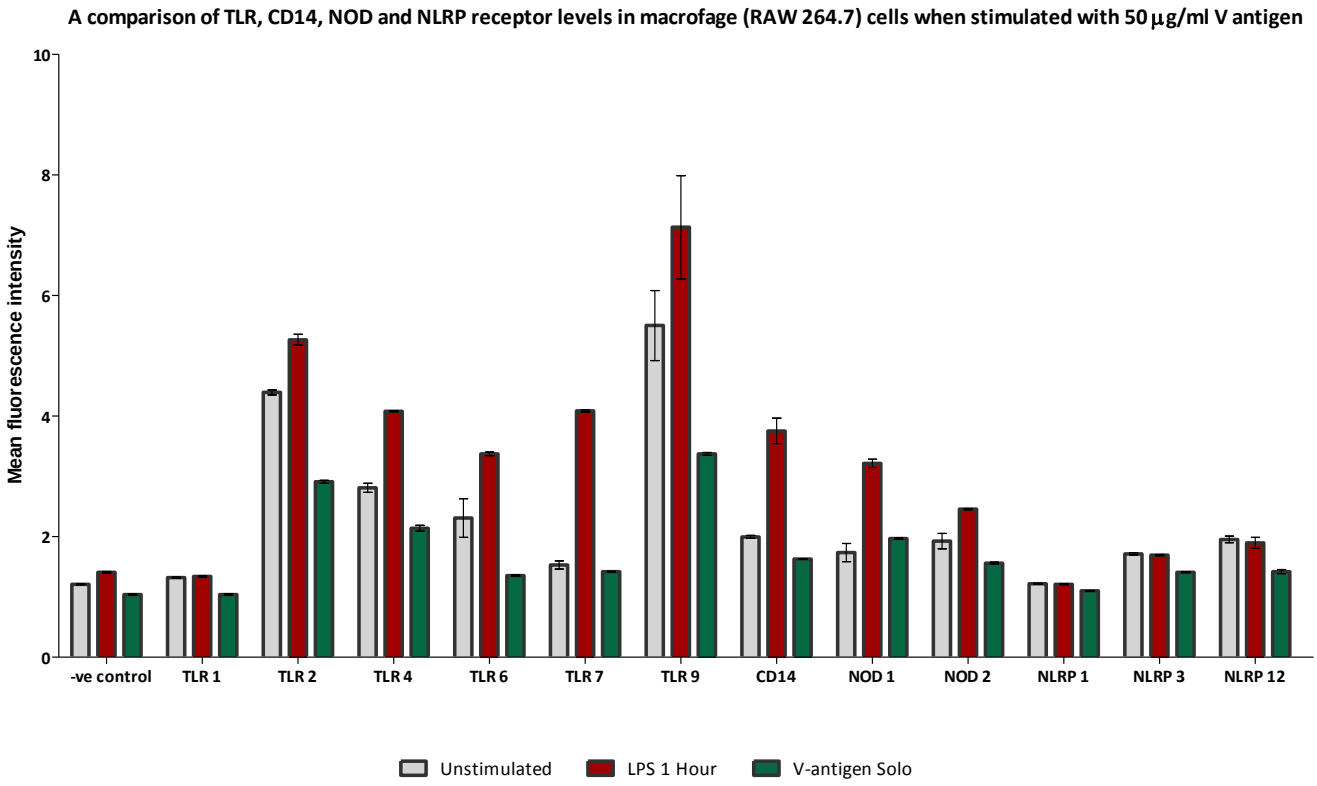


Figure 3.8: PRR expression levels on RAW 264.7 cells stimulated for 1 hour with 50µg/ml of V antigen. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on RAW 264.7 cells stimulated for 1 hour with 50µg/ml of V antigen. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur™ (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Subsequently, RAW 264.7 cells were pre-incubated for 1 hour with 50µg/ml of V antigen and stimulated for 1 hour with 100ng/ml of LPS (Figure 3.5). This pre-incubation caused a downregulation of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14 and NOD1. TLR1, TLR2, TLR4 and TLR6 expression levels decreased below the levels seen in unstimulated cells, i.e. basal expression levels found naturally in RAW 264.7 cells. In addition, TLR7, CD14 and NOD1 expression levels downregulated considerably, decreasing almost to the same expression level as unstimulated cells.

In contrast, the expression level of NOD2 seems not to change compared to cells stimulated solely with LPS. Furthermore, NLRP1, NLRP3 and NLRP12 showed an increase in expression, more prominently observed in NLRP12 expression level.

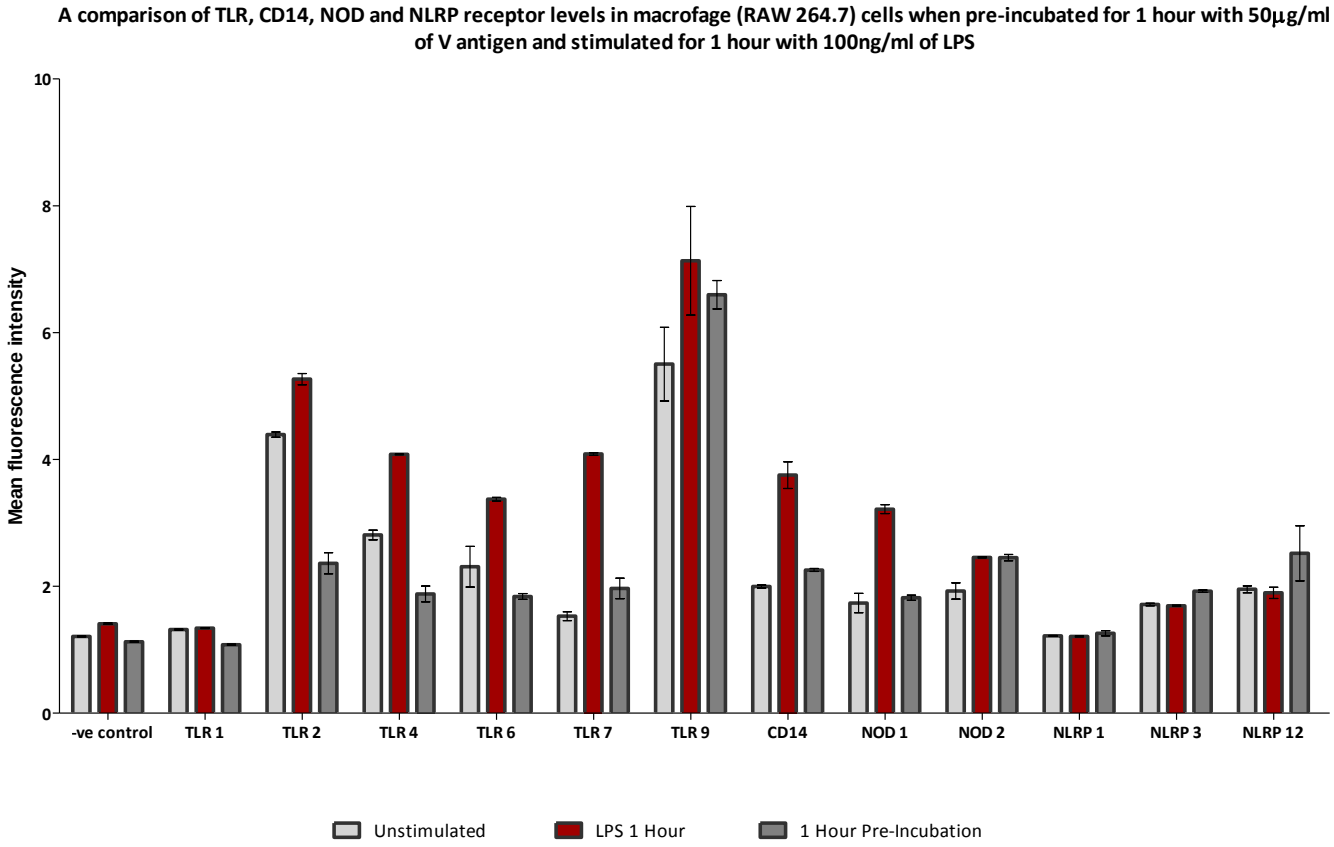


Figure 3.9: PRR expression levels on RAW 264.7 cells pre-incubated for 1 hour with 50µg/ml of V antigen and stimulated for 1 hour with 100ng/ml of LPS. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on RAW 264.7 cells pre-incubated for 1 hour with 50µg/ml of V antigen and stimulated for 1 hour with 100ng/ml of LPS. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

The following step was to examine what effect post-incubating RAW 264.7 cells with V antigen, after stimulating them with LPS, has on the expression of the different PRRs (Figure 3.10).

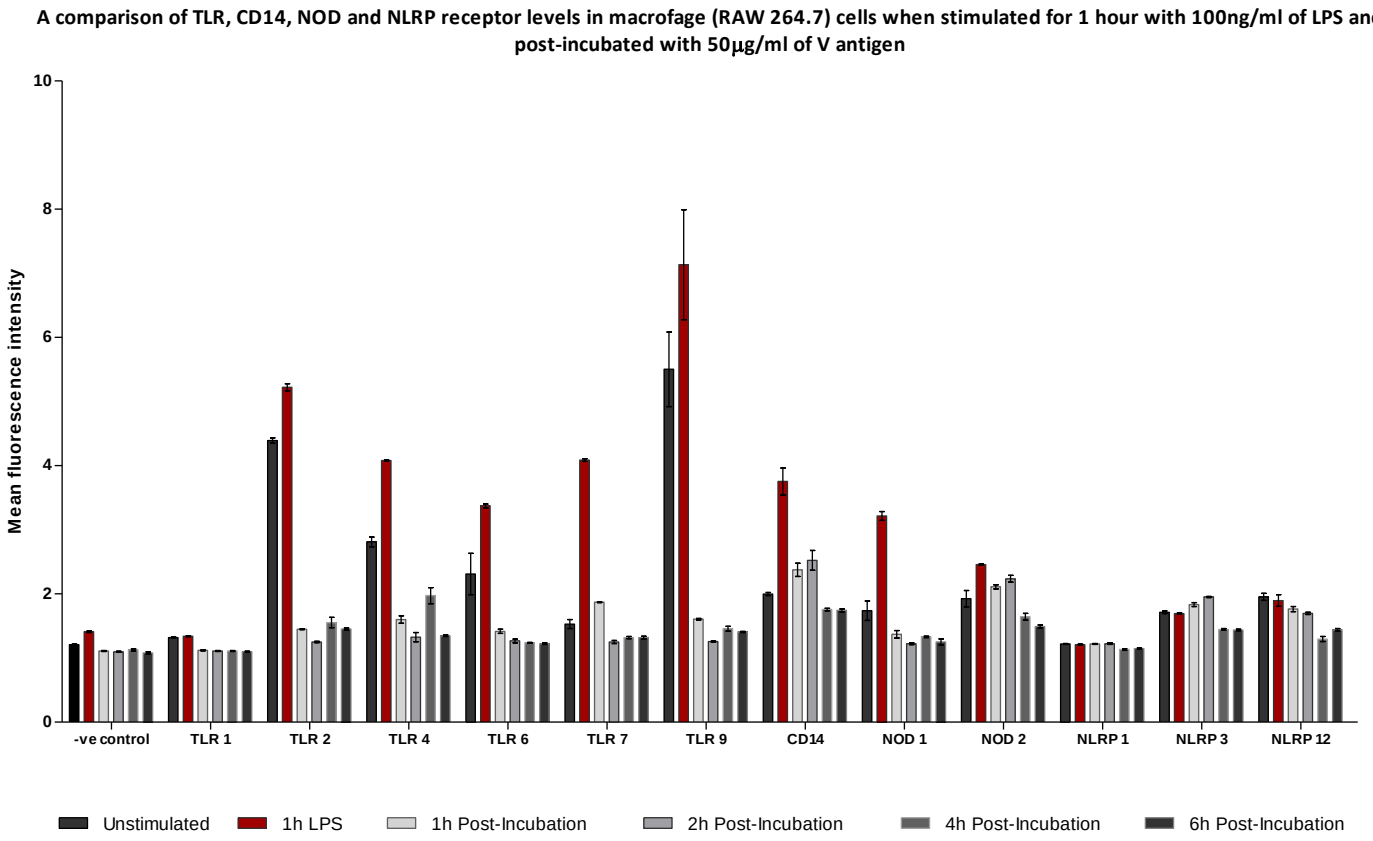


Figure 3.10: PRR expression levels on RAW 264.7 cells stimulated for 1 hour with 100ng/ml of LPS and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Receptor expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 on RAW 264.7 cells stimulated for 1 hour with 100ng/ml of LPS and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Unstimulated and isotype (-ve control) controls were performed. Cells were stimulated, fixed and labelled with a primary antibody against the receptor of interest, followed by an appropriate secondary antibody conjugated to FITC. Fluorescence was measured on a FACSCalibur^{TM} (Becton Dickinson), counting 10,000 cells per sample, not gated. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

When RAW 264.7 cells were stimulated with LPS and post-incubated with V antigen, all the PRRs investigated seemed to decrease expression levels, in comparison with cells stimulated with only with LPS. The PRRs that stand out the most in this downregulation due to post-incubation with V antigen are TLR2, TLR4, TLR6, TLR7, TLR9, CD14 and NOD1. The expression levels of these receptors seem to ultimately reduce to a lower level compared to unstimulated cells. Although, not as predominantly as the previously mentioned PRRs, NOD2, NLRP3 and NLRP3 expression levels also

seem to reduce down to as low or lower than the expression level seen in unstimulated cells. In contrast, TLR1 and NLRP1, in particular the later one, V antigen seems not to have an effect.

3.3.3. NF-κB and caspase-1 activation in RAW 264.7 cells in response to V antigen

In this study we also utilised RAW 264.7 cells to further elucidate whether V antigen could modulate NF-κB and caspase-1 activation in response to LPS, and consequently the secretion of inflammatory cytokines.

Experiments were performed in RAW 264.7 cells order to test the hypothesis that V antigen is able to modulate NF-κB and caspase-1 activation in response to LPS. The presence of phospho-IκB in the cell lysate indicates the transcription of pro-inflammatory cytokines. Similarly, the presence of caspase-1 is an indicative of inflammasome activation, which also leads to a secretion in inflammatory cytokines. Western blotting was utilised in order to determine the presence of both phospho-IκBα and caspase-1 p-10.

3.3.3.1 Presence of phospho-IκBα and caspase-1 p10 in RAW 264.7 cells in response to LPS

Initially, in order to determine whether LPS can lead to NF-κB and caspase-1 activation, RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. The supernatant was collected, at the respective time-points, frozen and stored for later cytokine assays. The cells were lysed and the samples were separated by size via SDS-PAGE (Section 2.9) and transferred onto a membrane (Section 2.10.1), which was probed for phospho-IκBα or caspase-1 p-10, with their respective primary antibody, followed by the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP). Membranes were imaged via enhanced chemiluminescence

(Section 2.11). It was shown that LPS could trigger NF-κB (Figure 3.19) and caspase-1 (Figure 3.20).

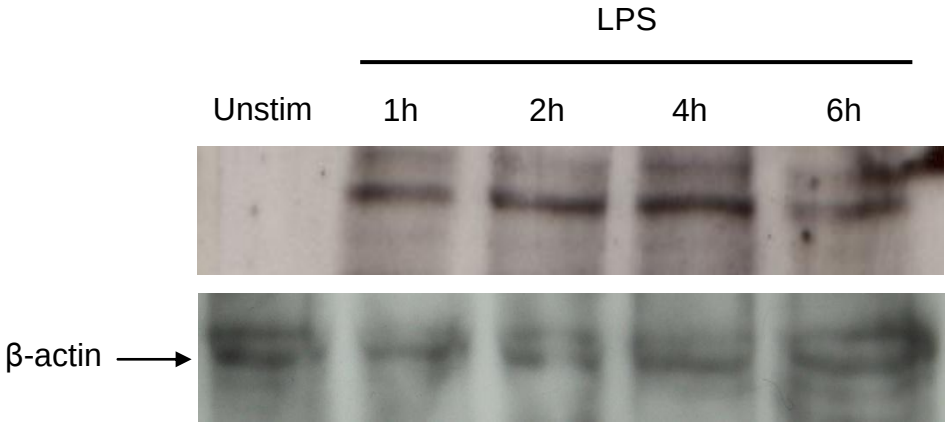


Figure 3.19: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

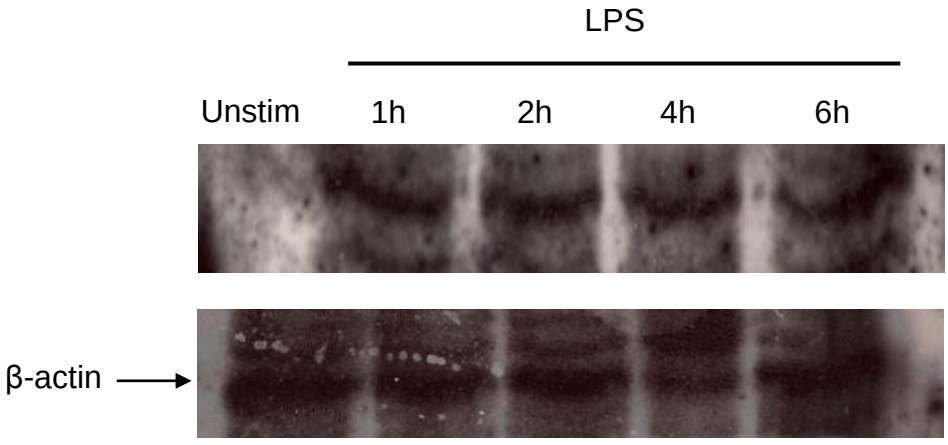


Figure 3.20: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

3.3.3.2. Presence of phospho-IκBα and caspase-1 p10 in RAW 264.7 cells in response to LPS pre- and post-incubated with V antigen

Once it was established that LPS is able to trigger NF-κB signalling cascade and caspase-1 activation in RAW 264.7 cells, whether V antigen was able to modulate this response was investigated. In order to do so, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. The cells were lysed and the samples were separated by size via SDS-PAGE (Section 2.9) and transferred onto a membrane (Section 2.10.1), which was probed for phospho-IκBα or caspase p-10, with their respective primary antibody, followed by the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP). Membranes were imaged via enhanced chemiluminescence (Section 2.11).

The results obtained from pre- and post-incubating with V antigen show that there is a still some activation of NF-κB in response to LPS, however it appears to be slightly inhibited. Although post-incubating with V antigen initially seems to show some NF-κB activation, when it hits the 4 hour and 6 hour post-incubation mark it seems to be inhibited (Figure 3.21).

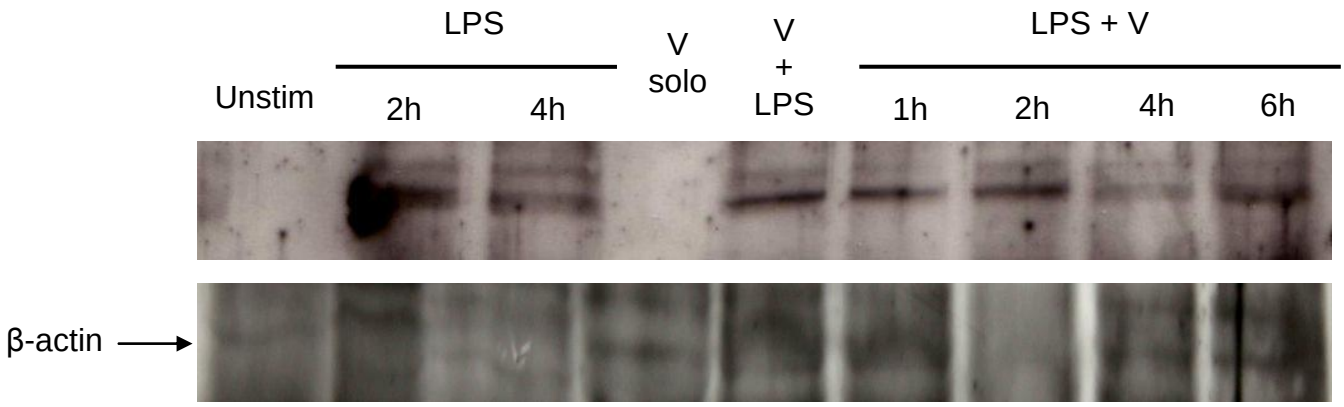


Figure 3.21: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells; stimulated for 2 and 4 hours with 100ng/ml LPS; stimulated for 1 hour with 50µg/ml of V antigen; pre-incubated for 1 hour with 50µg/ml of V antigen and subsequently stimulated for 1 hour with 100ng/ml LPS; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

Concerning caspase-1 activation, the results show that pre-incubation with V antigen seems not to have an effect on caspase-1 activation. On the contrary and post-incubating with V antigen results on a reduction of caspase-1. This reduction seems to gradually occur through the four post-incubation time-points, most prominent at 4 hour post-incubation (Figure 3.22).

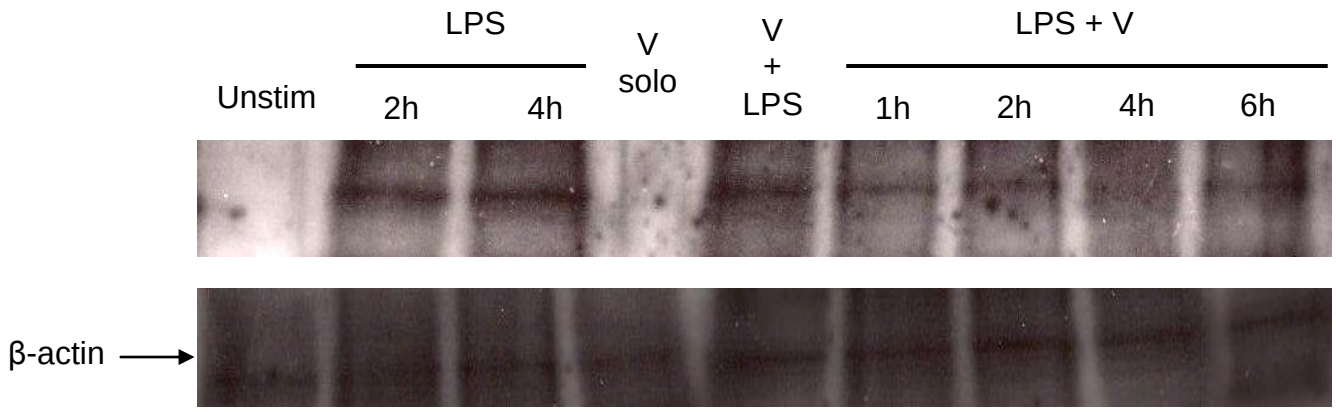


Figure 3.22: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells; stimulated for 2 and 4 hours with 100ng/ml LPS; stimulated for 1 hour with 50µg/ml of V antigen; stimulated for 1 hour with 50µg/ml of V antigen; pre-incubated for 1 hour with 50µg/ml of V antigen and subsequently stimulated for 1 hour with 100ng/ml LPS; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of V antigen. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

3.3.3.3. Cytokine secretion in RAW 264.7 cells in response to V antigen

Given that V antigen was found to modulate the expression of PRRs and the activation of both NF-κB and caspase-1 activation, the next step in this study was to examine the release of cytokines, the inflammatory mediators, caused by LPS and whether or not V antigen is able to modulate their secretion. Out of the inflammatory cytokines analysed (Interlukin-8 (IL-8), Interlukin-1β (IL-1β), Interlukin-6 (IL-6), Interlukin-10 (IL-10), Tumor Necrosis Factor (TNF) and Interlukin-12p70 (IL-12p70)), only the release of IL-1β, IL-6, IL-10 and TNF were significantly increased.

RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with 50µg/ml of V antigen for 1 hour. Additionally, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of V antigen. All stimulations were carried out in

25cm^2 flasks. The supernatants were collected post stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

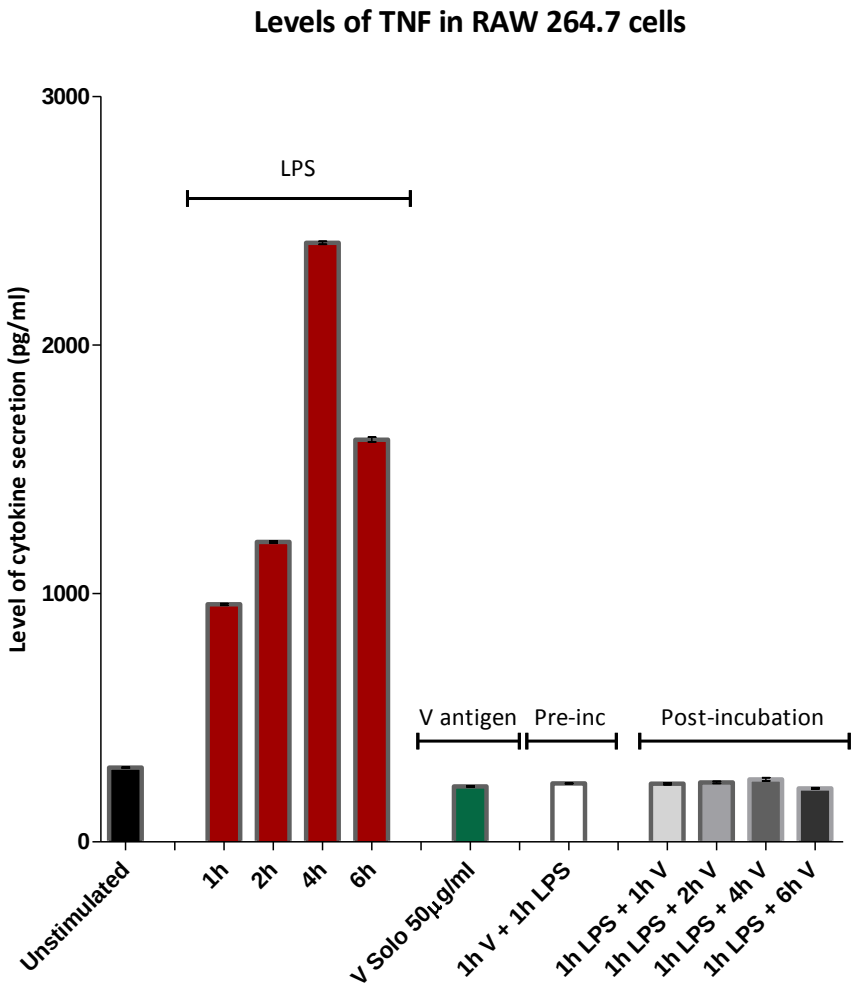


Figure 3.23: Secretion of TNF in RAW 264.7 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As expected, it was shown that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then decreased at the 6 hour mark. It was shown that V antigen on its own did not stimulate TNF secretion. TNF secretion was significantly down-regulated in response to pre- and post-incubation with V antigen, reaching unstimulated levels (Figure 3.23).

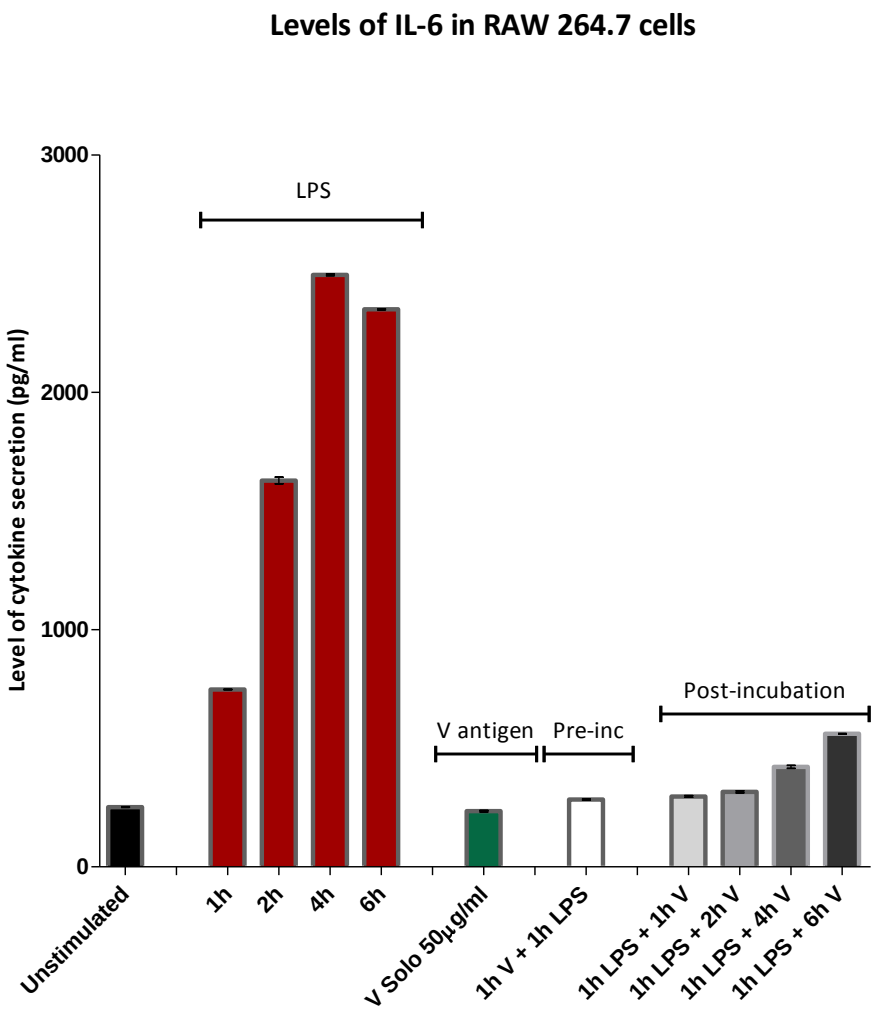


Figure 3.24: Secretion of IL-6 in RAW 264.7 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

When RAW 264.7 cells were stimulated with LPS, our results showed an increase in levels of IL-6, with a maximum by the 4 hour mark, followed by a decrease in secretion at the 6 hour time-point. When cells were stimulated with 50µg/ml of V antigen by itself our results showed no change in IL-6 production in comparison with unstimulated cells. The same can be said to THP-1 cells pre-incubated with V antigen. The first 2 post-incubation time-points with V antigen showed that the levels of IL-6 were alike with the levels of unstimulated cells. Our results showed a slight increase in IL-10 secretion when cells were post-incubated with V antigen for 4 and 6 hours compared to unstimulated cells, although much lower levels compared with cells stimulated with LPS (Figure 3.24).

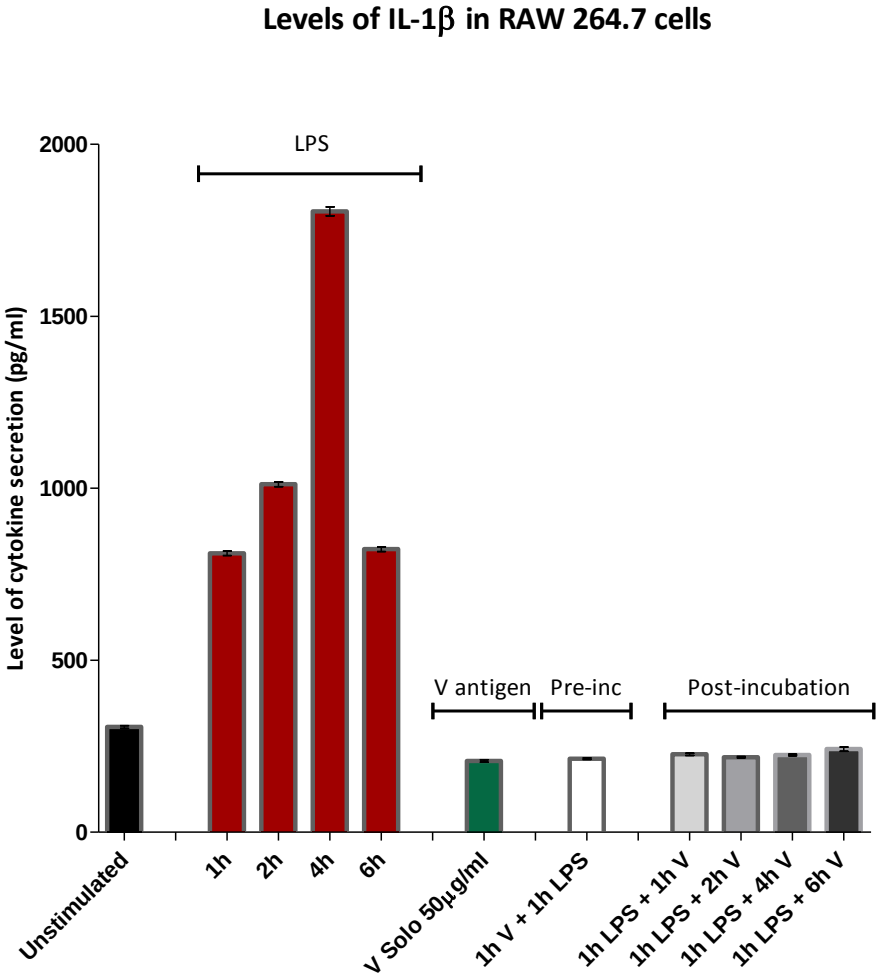


Figure 3.25: Secretion of IL-1β in RAW 264.7 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results show that when RAW 264.7 cells were stimulated with LPS the levels of IL-1β increased. With regards to cells stimulated solely with V antigen and pre or post-incubated with V, the levels of IL-1β are lower compared to the levels seen in unstimulated cells (Figure 3.25).

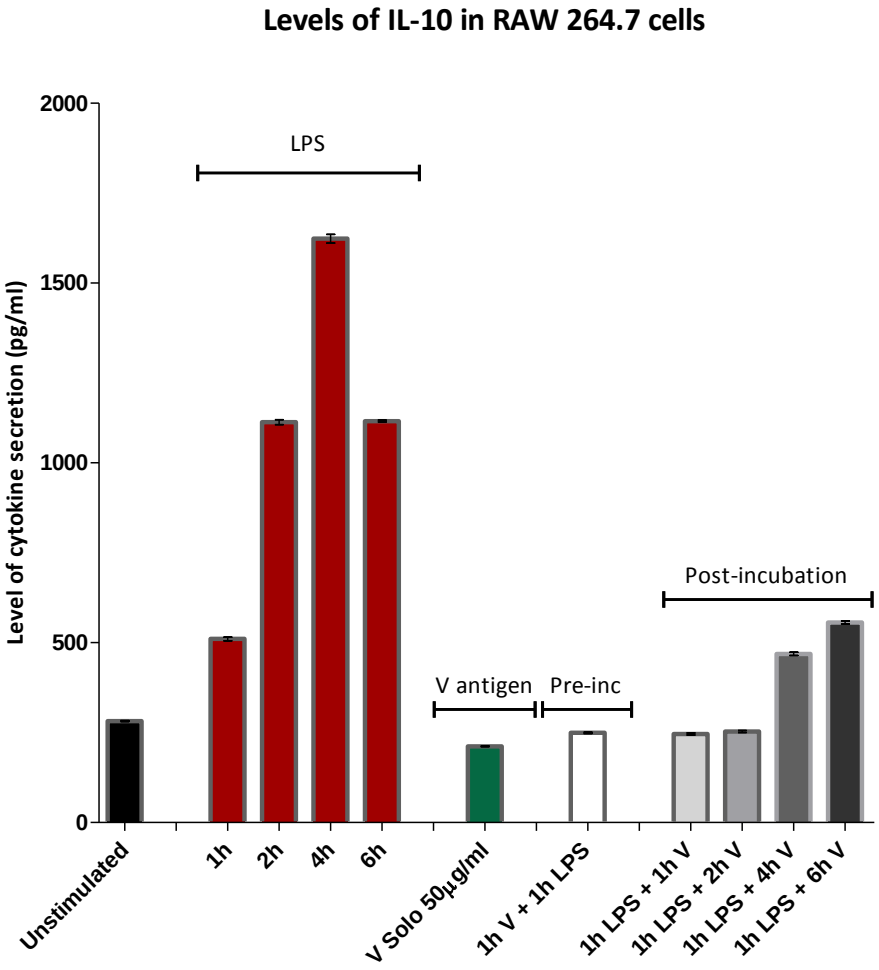


Figure 3.26: Secretion of IL-10 in RAW 264.7 cells in response to LPS, V antigen and pre- or post-incubation with V antigen. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V antigen for 1 hour; pre-incubated with 50µg/ml V antigen for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V antigen. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

When RAW 264.7 cells were stimulated with LPS they gradually secreted IL-10 in response to LPS, with a maximum at the 4 hour stimulation, followed by a downregulation at the 6 hour mark. RAW 264.7 cells stimulated with V antigen by itself did not result in IL-10 production. Similarly, pre-incubating THP-1 cells with V antigen did not result in a production of IL-10. The first two post-incubation time-points showed no increase in IL-10 production, whereas 4 and 6 hour post-incubation with V antigen

showed a slight increase in IL-10 production, although only matching the levels seen in cells stimulated with LPS for 1 hour (Figure 3.26).

3.3.4. Conclusion

In addition to THP-1 cells, RAW 264.7 cells were also utilised in order to elucidate the effects of V antigen on the innate immune responses. Experiments with a similar sequential layout to THP-1 were used to investigate the effects of V antigen on RAW 264.7 cell PRRs. In RAW 264.7 cells, LPS caused up-regulation of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1 and NOD2. Moreover, but not as acute, NLRP1, NLRP3 and NLRP12 expression levels increased in response to LPS. When RAW 264.7 cells were stimulated with V antigen TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12 expression levels were lowered in comparison with cells stimulated just with LPS. NLRP1 showed a slight decrease in its expression level in response to V antigen.

When RAW 264.7 were pre-incubated with V antigen, the expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14 and NOD1 lowered. In contrast, the expression level of NOD2 did not seem change and NLRP1, NLRP3 and NLRP12 showed a very slight increase in expression, more prominently observed in NLRP12 expression level. Post-incubating RAW cells with V antigen caused a downregulation of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 – an overall decrease in expression levels, across the four post-incubation time-points, of all PRRs investigated in this study.

Since our results showed that V antigen was able to modulate the expression of different PRRs in RAW 264.7 cells the next step was to test the hypothesis that V antigen is able to modulate NF-κB and caspase-1 activation, and as a consequence the release of inflammatory cytokines.

Initially, we tested if both THP-1 and RAW 264.7 cells when stimulated with LPS would activate NF-κB and caspase-1. Our results showed that both cell types do activate both NF-κB and caspase-1. Once it was established that LPS was able to trigger NF-κB signalling cascade and caspase-1, whether V antigen was able to modulate this response was investigated. Our results revealed that RAW 264.7 cells V antigen slightly inhibited NF-κB activation and gradually decreased caspase-1 activation over time.

With reference to inflammatory cytokine release, V antigen seemed to inhibit completely TNF secretion in both cell types. Similarly, IL-1β secretion levels seem to be inhibited in RAW 264.7 cells in response to V antigen. IL-6 levels seem to be downregulated down to basal levels in RAW 264.7 cells in response to V antigen. In the case of IL-10 RAW 264.7 cells showed no increase in IL-10 production in the first two post-incubation time-points, whereas 4 and 6 hour post-incubation with V antigen showed a slight increase in IL-10 production, although only matching the levels seen in cells stimulated with LPS for 1 hour.

Overall our data suggests that V antigen is able to modulate PRR expression as well as inhibit signalling cascades and pro-inflammatory cytokine secretion in RAW 264.7 cells. When compared to THP-1 cells the results are similar, but rather more pronounced inhibition is seen in RAW 264.7 cells.

3.4. Functional domain of V antigen

Previous studies have shown that, not only the entire, but also small regions of *Yersinia pestis* V antigen can provide protection against the plague. It has been suggested that *Y. pestis* V antigen contains a number of protective epitopes that are located in a central region between amino acids (a.a.) 135 and 275 (Hill *et al.*, 1997). Vernazza *et al.* (2009) aimed to identify the minimum protective fraction of *Y. pestis* V antigen that could provide protection against plague. In order to do so, they generated small regions of the V antigen, residing between the central a.a. 135-275 fragment.

In order to investigate the functional domain of V antigen that is responsible for the immunomodulatory effects we observed, V antigen fractions had to be produced. V antigen fractions were cloned in *E. coli* and kindly supplied to us by Dr. Claire Vernazza, Ministry of Defence, Porton Down. In order to purify the V antigen fractions, the *E. coli* strain had to be cultured in Luria broth and the protein isolated using affinity chromatography.

V antigen fragments were selected according to previous studies performed by Vernazza et al. (2009) (Table 2.2). In this study the V antigen fragments selected were V1, V3, V4 and V5.

Fragment	Deletion type	Amino acids
V1	**-**	**135-275**
V2	N-terminal	168-275
V3	**N-terminal**	**175-275**
V4	**C-terminal**	**135-268**
V5	**C-terminal**	**135-262**
V6	Internal	135-275 Δ218-234

Table 2.5: V antigen fragments. In this study the V antigen fragments selected were V1, V3, V4 and V5 (highlighted). Adapted from Vernazza et al. (2009).

3.4.1. Purification of V antigen fractions V1, V3, V4 and V5

In order to produce V antigen fractions (V1, V3, V4 and V5), the recombinant protein plasmids (pGEX6P-1) with a glutathione S-transferase (GST) tag was used which was kindly provided by Dr. Claire Vernazza (Defence Science and Technology Laboratory, Porton Down, Salisbury, UK). The plasmids were transformed into *E. coli* BL21 cells as previously described by Carr et al. (1999).

The cloning and expression of the genes was done by Claire Vernazza and colleagues as described in Vernazza et al. (2009). Briefly, the *lcrV* fragments gene was amplified by PCR from the chromosomal DNA of *Y. pestis* strain GB. Primers: 135 5' CGGATCCATTTTGAAAGTGATTGTTG 3', 175 3' CGGATCCATTAAGCATCTGTCT-AGTA 5', 262 5' CGTCGACTCAATCTTTATTATAAGAG 3', 268 5' CGTCGACTCAG-TGAGATAATTCATTA 3', 275 5' CGTCGACTCAATCCGAGCAGGTGGTG 3' were cloned into a pGEX6P-1 (GE Healthcare) to make the recombinant plasmids, pGEX6P-1.

E. coli BL21 cells containing the recombinant plasmid were cultured in Luria broth containing 100mg/ml ampicillin. Cultures were placed in a 37ºC rotary incubator at 120RPM until an A_{600} of 0.3 was achieved; cultures were then induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 6 hours and harvested by centrifugation at 7000RPM for 30 minutes (Section 2.5.2).

Harvested cells were resuspended in PBS and lysed by repeated freeze-thaw cycles. Crude cell extract was clarified by centrifugation at 2500RPM for 30 minutes and the supernatant added to glutathione Sepharose columns, previously equilibrated with PBS. Unbound material was washed off with PBS. Cleavage buffer (10ml) (50mM Tris/150mM NaCl/1mM EDTA/1mM DTT/0.01%) with PreScission™ Protease (150µl) was added to the column and incubated at room temperature for 3 hours. The column

was then washed with more cleavage buffer (20ml) and the elution was collected (Section 2.5.5).

The elution was concentrated and subsequently passed through a Profos EndoTrap® blue 10 to ensure there was no LPS in the final solution (Section 2.5.7.1). The HyCult LAL Chromogenic Endotoxin Quantitation Kit was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* Amebocyte Lysate (LAL) assay, which was found to be none (Section 2.5.8).

3.4.2. Effects of V antigen fractions on innate immune signalling

As previously mentioned, one of the consequences of activation of TLRs is the expression of genes via NF-κB which, normally, is sequestered by unphosphorylated IκB. One of the most common signalling pathways from TLR activation leads to the phosphorylation of IκB and consequently the release of NF-κB, which in turn lead to the production of inflammatory cytokines. Furthermore, also mentioned above, inflammasome sensor molecules, such as NODs and NLRPs, can activate caspase-1 where its main role is to convert pro-IL-1β and pro-IL-18 into their bioactive cytokine IL-1β and lL-18, respectively.

The protein complex NF-κB is the transcription factor that is involved in the cellular response to stress and, of particular interest to this work, presence of cytokines. The presence of phospho-IκB in the cell lysate indicates the transcription of pro-inflammatory cytokines. Similarly, the presence of caspase-1 p10 is an indicative of inflammasome activation, which also leads to a secretion in inflammatory cytokines. Western blotting was utilised in order to determine the presence of both phospho-IκBα and caspase-1 p-10.

In order to determine whether V antigen fractions were able to modulate this response RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS or stimulated for 1 hour with 100ng/ml of LPS and then post-incubated for 1, 2, 4 and 6

hours with the different fractions. The supernatant was collected, at the respective time-points, frozen and stored for later cytokine assays. The cells were lysed and the samples were separated by size via SDS-PAGE (Section 2.9) and transferred onto a membrane (Section 2.10.1), which was probed for phospho-IκBα or caspase p-10, with their respective primary antibody, followed by the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP). Membranes were imaged via enhanced chemiluminescence (Section 2.11).

In this study, out of the six fragments that possibly could confer protection, proposed by Vernazza et al. (2009), four were chosen – V1, V3, V4 and V5 (Figure 3.27). V1 was selected because it has been previously shown to be protective against plague, V3 due to a N-terminal deletion whereas V4 and V5 a C-terminal deletion type.

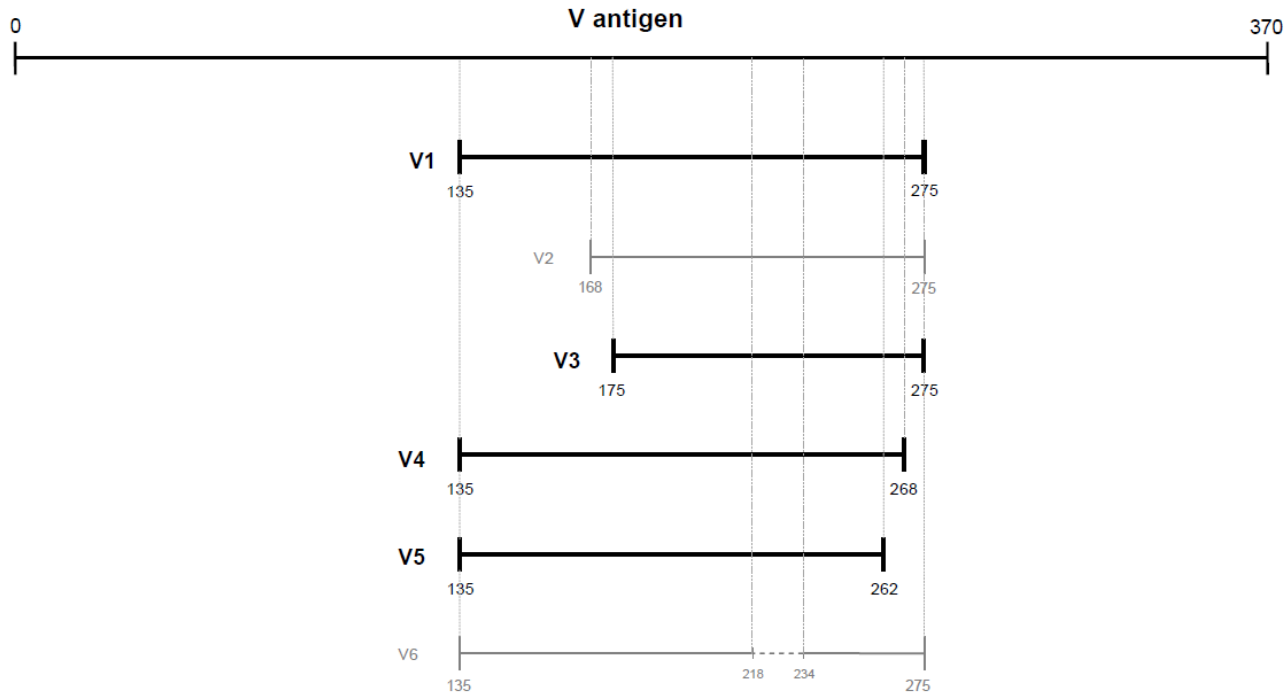


Figure 3.27: Schematic representation of V antigen and its fragments V1, V2, V3, V4, V5 and V6. In this study, the fragments selected were V1 (a.a. 135-275), V3 (a.a. 175-275), V4 (a.a. 135-268) and V5 (135-262).

The results obtained from post-incubating RAW 264.7 cells with V1 fraction show that there is a minor activation of NF-κB in response to LPS, however it appears to majorly inhibited compared to cells stimulated solely with LPS. At the 6 hour post-incubation NF-κB activation seems to increase somewhat (Figure 3.28). Post-incubating RAW 264.7 cells with V1 fraction show that there is a total inhibition of caspase-1 activation (Figure 3.29).

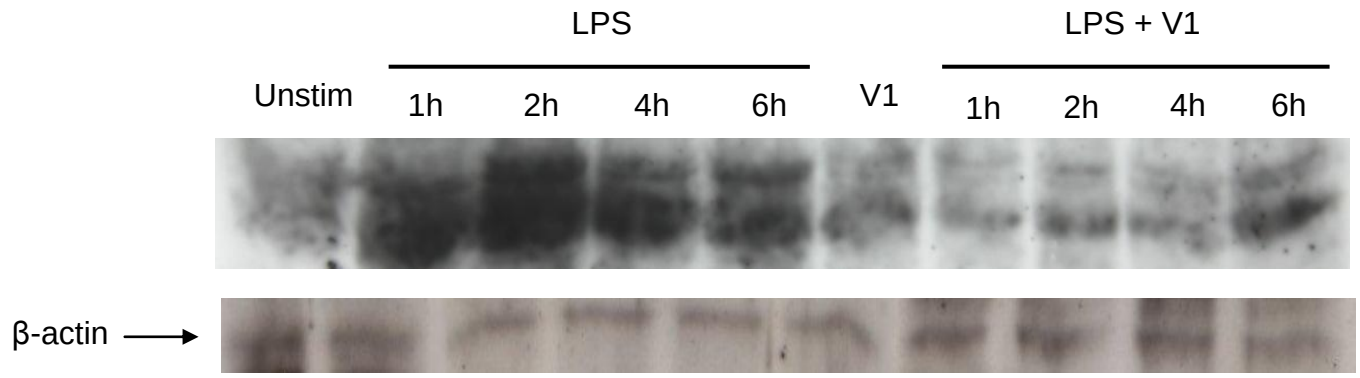


Figure 3.28: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V1; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V1. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

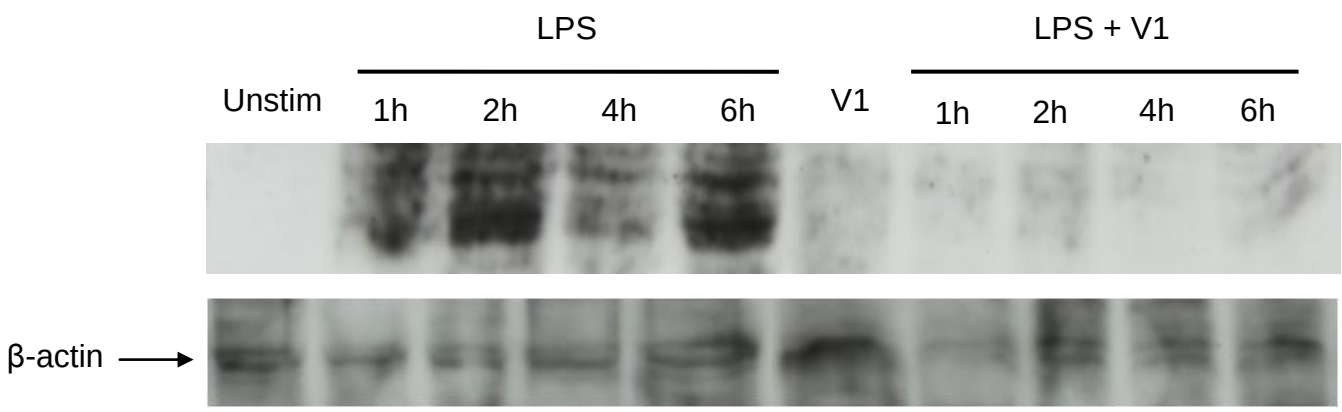


Figure 3.29: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V1; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V1. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

Concerning V3 fraction, the results show that post-incubations result on a small inhibition of NF-κB compared to RAW 264.7 cells stimulated with just LPS. This inhibition seems to gradually occur through the first three post-incubation time-points, with a considerable surge in NF-κB at the 6 hour mark (Figure 3.30). With regards to caspase-1 activation, V3 seems to downregulate its activation at the first hour of post-incubation and at the second hour it caspase-1 activation seems to increase. A re-inhibition seems to occur for the remaining post-incubation time-points (Figure 3.31).

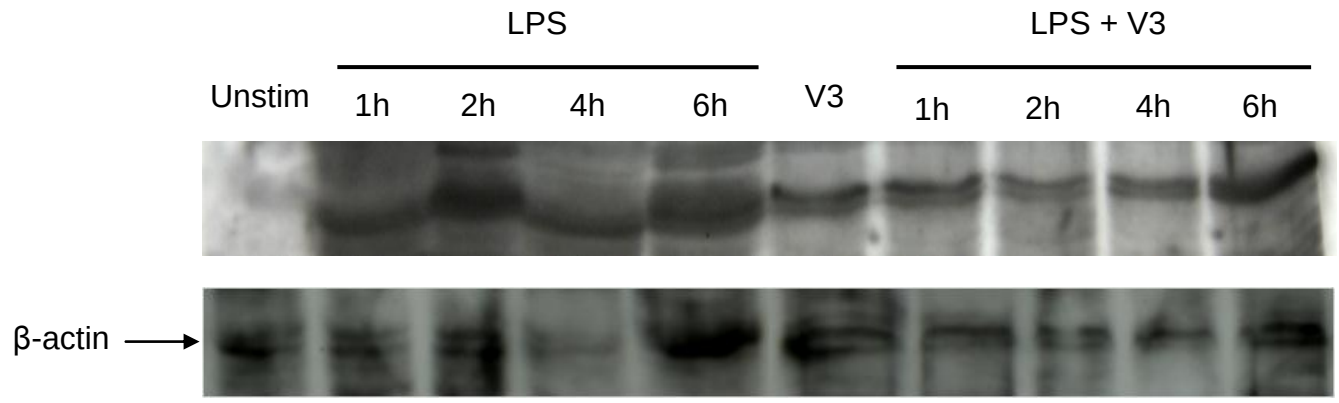


Figure 3.30: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V3; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V3. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

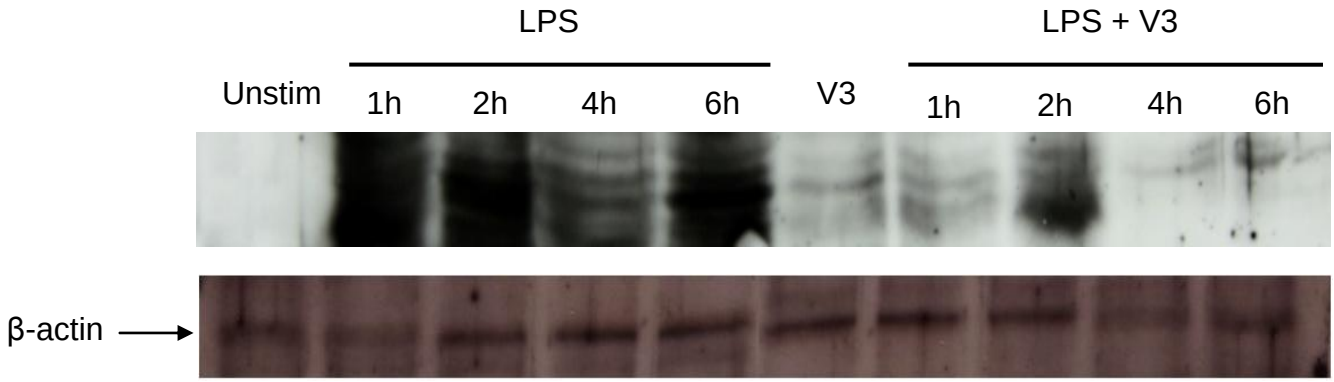


Figure 3.31: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V3; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V3. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

The results obtained from post-incubating RAW 264.7 cells with V4 fraction show that there is a total inhibition of NF-κB activation (Figure 3.32). Likewise, post-incubating RAW 264.7 cells with V1 fraction show that there is a complete inhibition of caspase-1 activation (Figure 3.33).

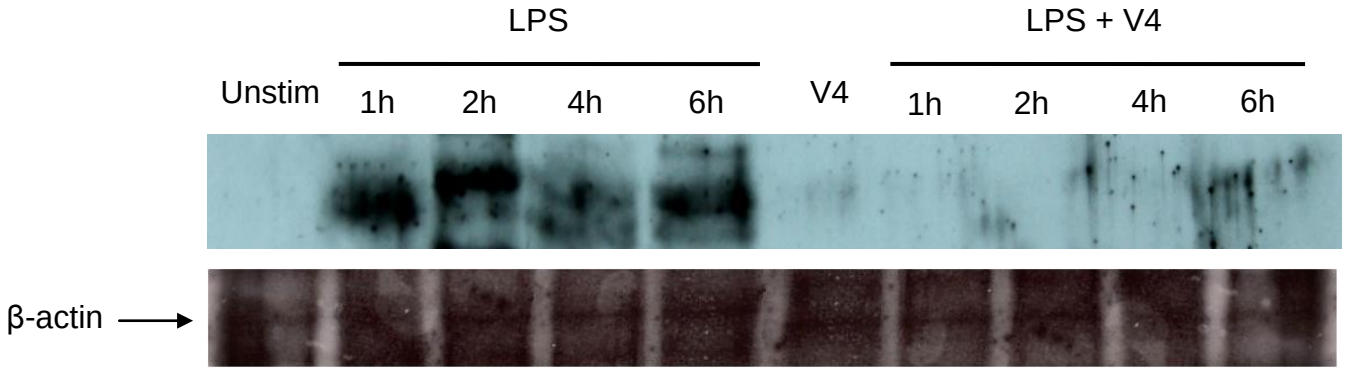


Figure 3.32: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V4; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V4. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

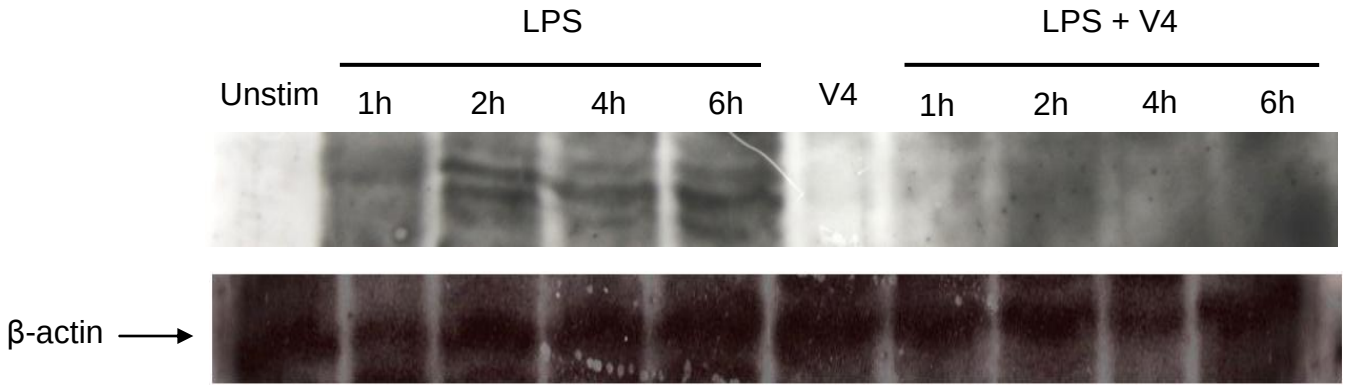


Figure 3.33: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V4; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V4. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

Regarding the V5 fraction, the results show that post-incubation seems to predominantly inhibit NF-κB activation (Figure 3.34). In the same way, V5 fraction seems to have the same effect on caspase-1 activation, as it seems to entirely downregulate it (Figure 3.35).

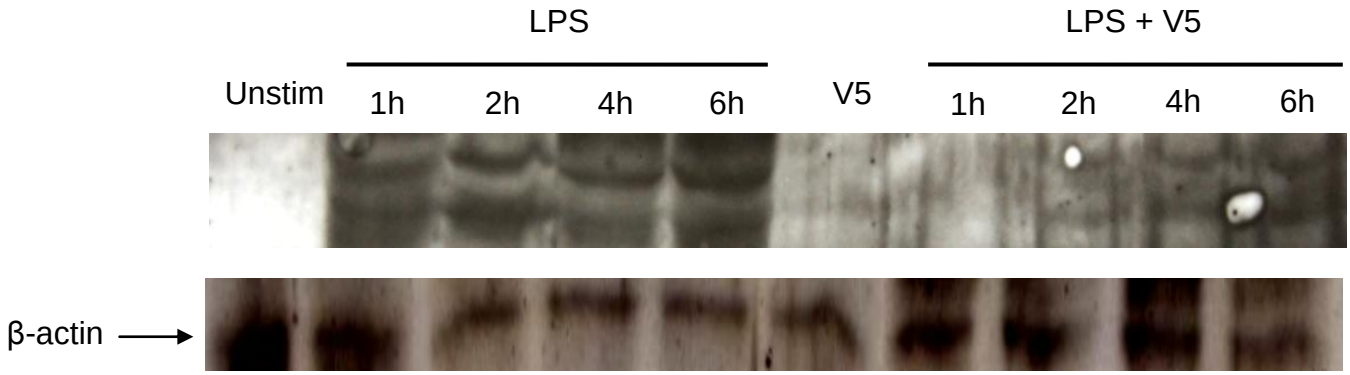


Figure 3.34: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V5; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V5. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

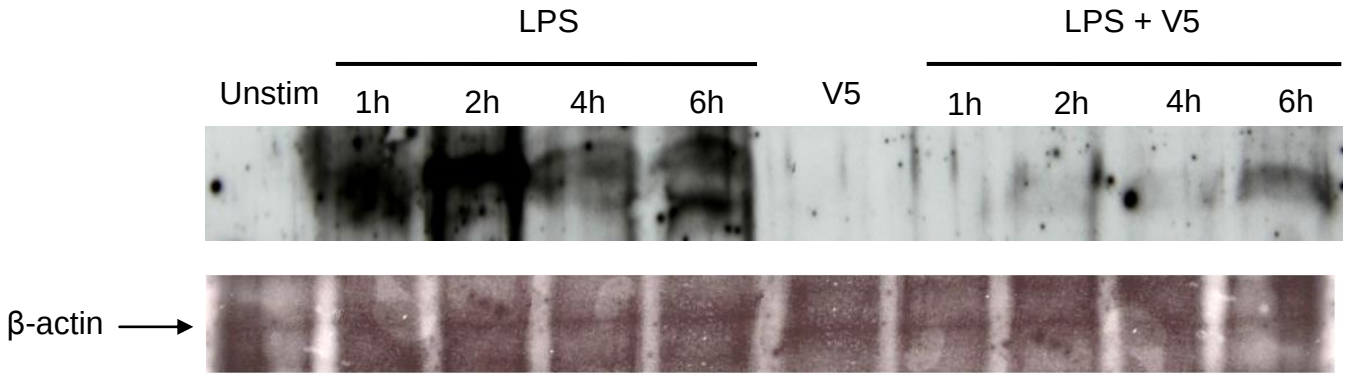


Figure 3.35: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; stimulated with for 1 hour with 50µg/ml of fragment V5; and stimulated for 1 hour with 100ng/ml LPS and subsequently post-incubated for 1, 2, 4 and 6 hours with 50µg/ml of fragment V5. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

In this study we also utilised pre-incubated RAW 264.7 cells with the different V fractions to further elucidate the effect they have in NF-κB and caspase-1 activation in response to LPS. It seems that pre-incubating RAW264.7 cells with the different V fractions has an overall NF-κB inhibitory effect. It is more noticeable in V1 then V4 and V5. V3 seems to also have an inhibitory effect on NF-κB activation, although not as V1, V4 or V5 (Figure 3.36). With regards to caspase-1 activation, V1 appears to have the highest effect in inhibiting it. On the other hand, V3, V4 and V5, appear to slightly inhibit caspase-1 activation, but not to the same extent as V1 (Figure 3.37).

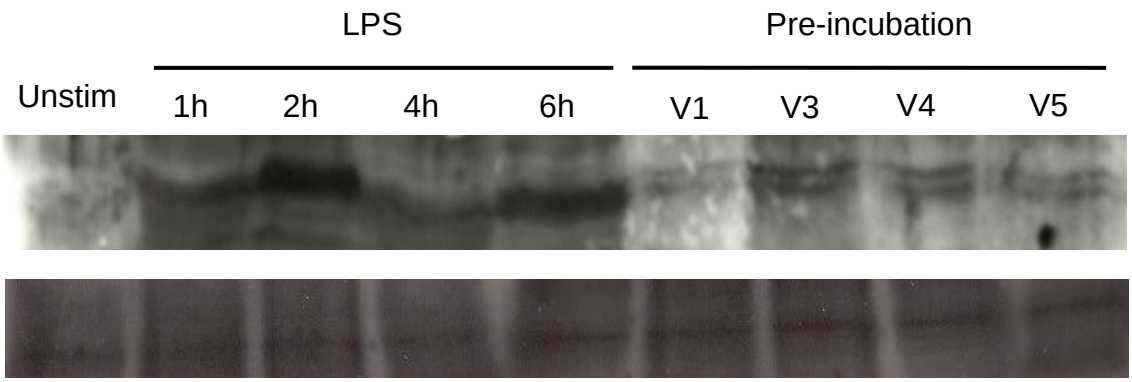


Figure 3.36: Western blot of phospho-IκBα from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; and pre-incubated for 1 hour with 50µg/ml of fragments V1, V3, V4 or V5 and subsequently stimulated for 1 hour with 100ng/ml of LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for phospho-IκBα followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

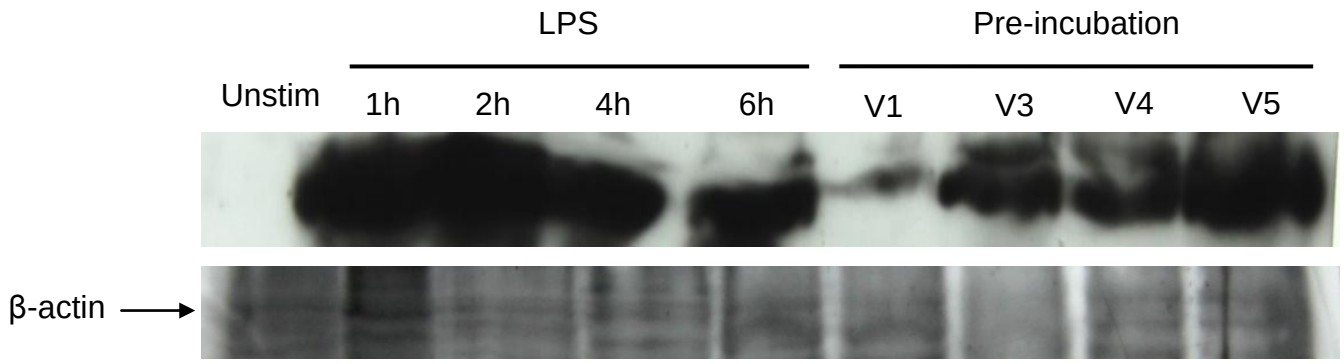


Figure 3.37: Western blot of caspase-1 p10 from lysates of unstimulated RAW 264.7 cells and those stimulated for 1, 2, 4 and 6 hours with 100ng/ml LPS; and pre-incubated for 1 hour with 50µg/ml of fragments V1, V3, V4 or V5 and subsequently stimulated for 1 hour with 100ng/ml of LPS. Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with an antibody specific for caspase-1 p10 followed by the appropriate secondary antibody conjugated to HRP. Enhanced chemiluminescence was used for detection. β-actin was used as a loading control.

3.4.3. Cytokine secretion in RAW 264.7 cells in response to V antigen fractions

Our results show that V antigen fractions had an effect on the activation of both NF-κB and caspase-1 activation. The following step in this study was to examine the release of cytokines, the inflammatory mediators, caused by LPS and whether or not the different V antigen fractions were able to modulate their secretion. Out of the inflammatory cytokines analysed (Interlukin-8 (IL-8), Interlukin-1β (IL-1β), Interlukin-6 (IL-6), Interlukin-10 (IL-10), Tumor Necrosis Factor (TNF) and Interlukin-12p70 (IL-12p70)), only the release of IL-1β, IL-6, IL-10 and TNF were significantly increased.

3.4.3.1. Cytokine secretion in RAW 264.7 cells in response to V1

RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with 50µg/ml of the V1 for 1 hour. Additionally, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of the V1. All stimulations were carried out in 25cm^2 flasks. The supernatants were collected post stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

Out of the inflammatory cytokines analysed, only the release of TNF, IL-6, IL-1β and IL-10 were significantly increased in response to V1.

As expected, it was shown that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then decreased at the 6 hour mark. It was shown that V1 on its own did not stimulate TNF secretion. TNF secretion was significantly down-regulated in response to pre- and post-incubation with V1 fraction, reaching as little as unstimulated levels (Figure 3.38).

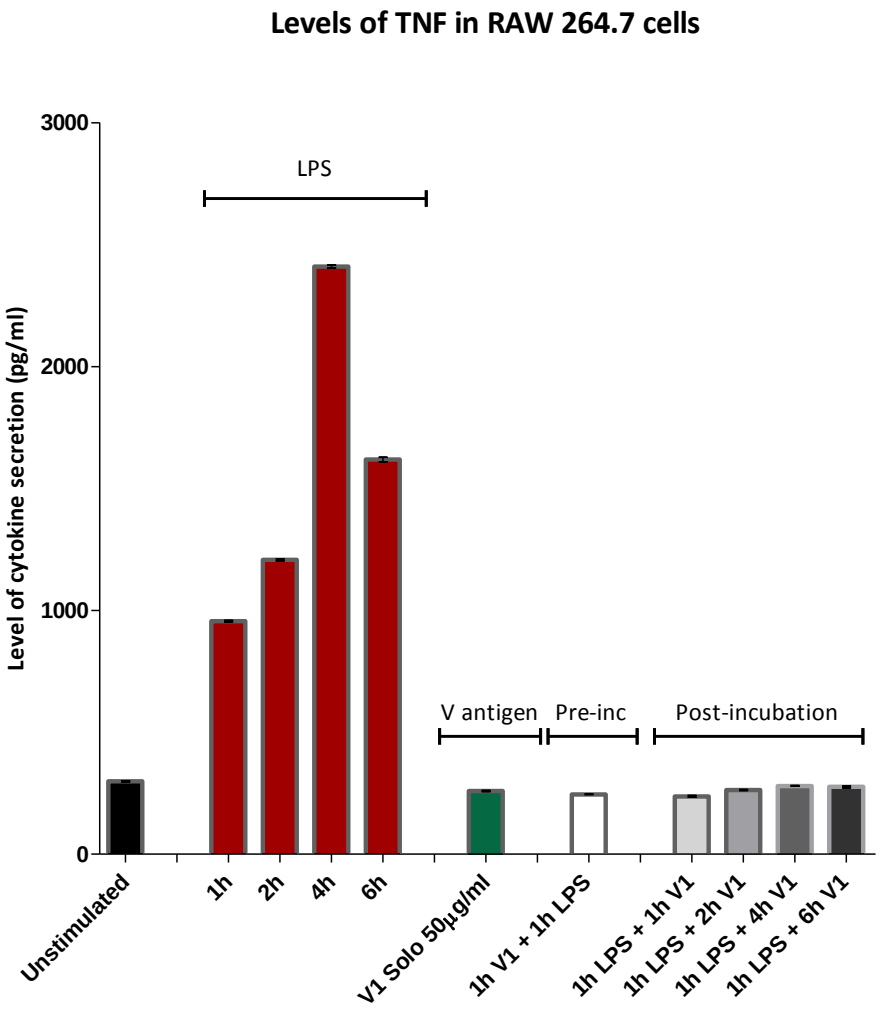


Figure 3.38: Secretion of TNF in RAW 264.7 cells in response to LPS, V1 and pre- or post-incubation with V1. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V1 for 1 hour; pre-incubated with 50µg/ml V1 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V1. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results showed that LPS stimulated the release of IL-6. Its release increased over the first 4 hours and then slightly lowered at the 6 hour mark. It was shown that V1 on its own did not stimulate IL-6 secretion. IL-6 secretion was significantly dampened in response to pre- and post-incubation with V1 fraction, reaching as little as unstimulated levels (Figure 3.39).

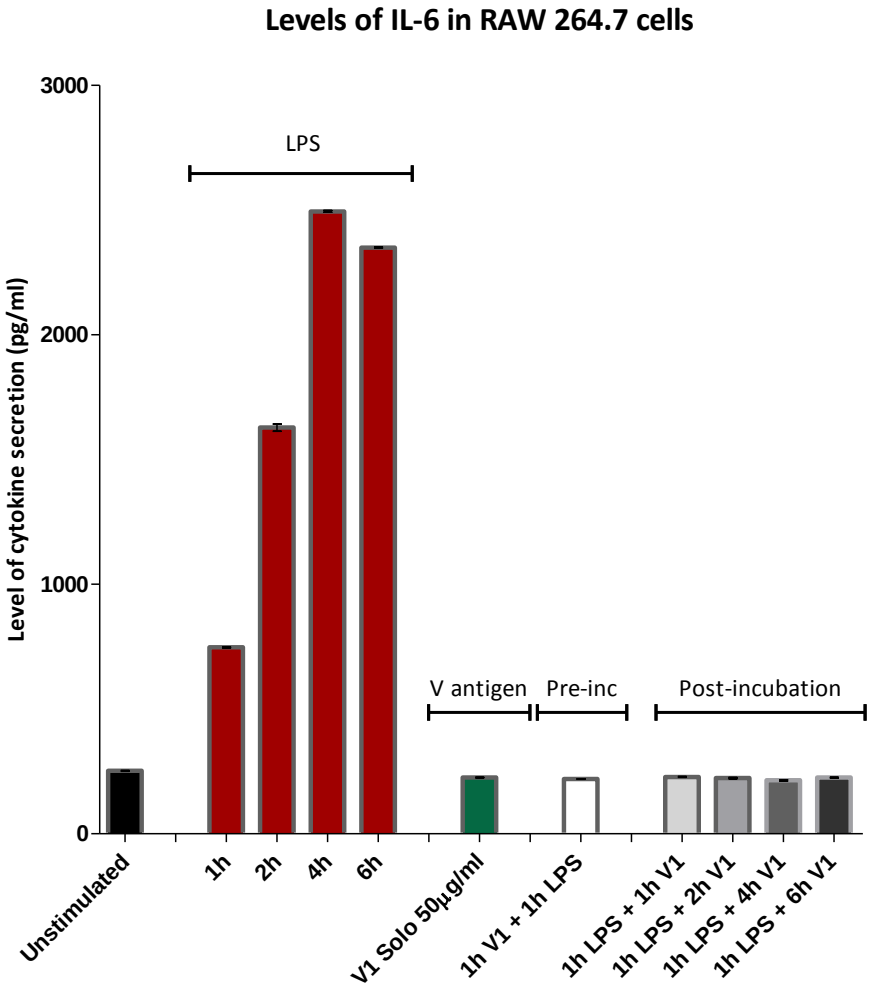


Figure 3.39: Secretion of IL-6 in RAW 264.7 cells in response to LPS, V1 and pre- or post-incubation with V1. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V1 for 1 hour; pre-incubated with 50µg/ml V1 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V1. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As anticipated, it was shown that LPS stimulated the release of IL-1β. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V1 by itself did not stimulate IL-1β secretion. IL-1β secretion was reduced in response to pre- and post-incubation with V1 fraction, reaching as little as unstimulated levels (Figure 3.40).

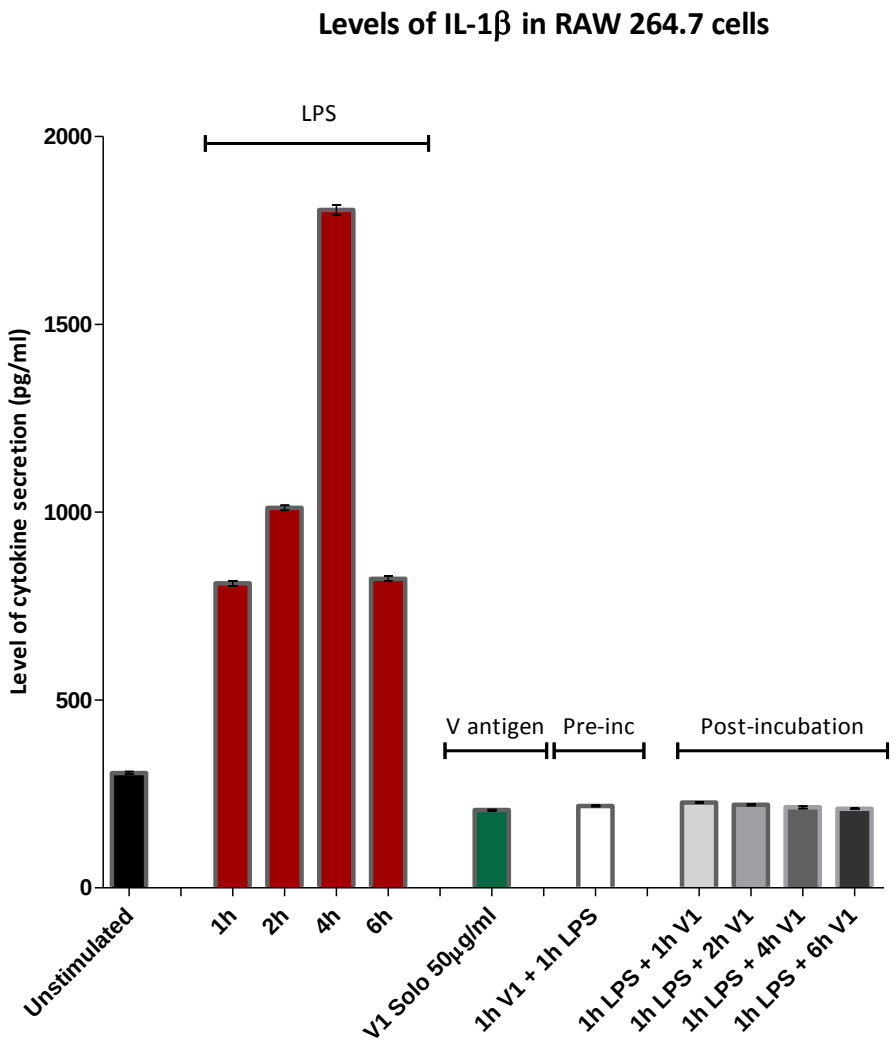


Figure 3.40: Secretion of IL-1β in RAW 264.7 cells in response to LPS, V1 and pre- or post-incubation with V1. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V1 for 1 hour; pre-incubated with 50µg/ml V1 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V1. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results showed that LPS stimulated the release of IL-10. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V1 by itself did not stimulate IL-10 secretion. IL-10 secretion was reduced in response to pre- and the first two post-incubation time-points with V1 fraction, reaching as little as unstimulated levels. Our results showed a slight increase in IL-10 secretion when cells were post-

incubated with V1 for 4 and 6 hours compared to unstimulated cells, although much lower levels compared with cells stimulated with LPS (Figure 3.41).

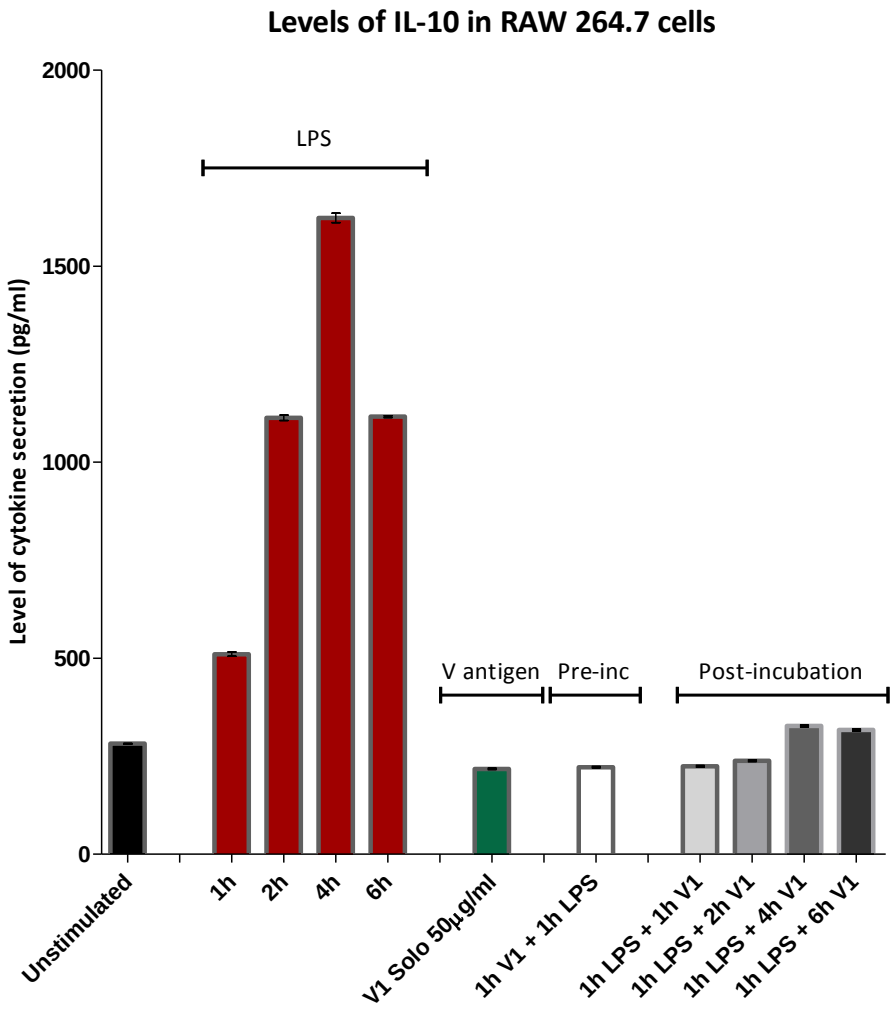


Figure 3.41: Secretion of IL-10 in RAW 264.7 cells in response to LPS, V1 and pre- or post-incubation with V1. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V1 for 1 hour; pre-incubated with 50µg/ml V1 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V1. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

3.4.3.2. Cytokine secretion in RAW 264.7 cells in response to V3

RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with 50µg/ml of the V1 for 1 hour. Additionally, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of the V1. All stimulations were carried out in 25cm^2 flasks. The supernatants were collected post stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

Out of the inflammatory cytokines analysed, only the release of TNF, IL-6, IL-1β and IL-10 were significantly increased in response to V3.

As predicted, our results show that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V3 by itself did not stimulate TNF secretion. Our results showed a slight increase in TNF secretion when cells were pre- or post-incubated with V3 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.42).

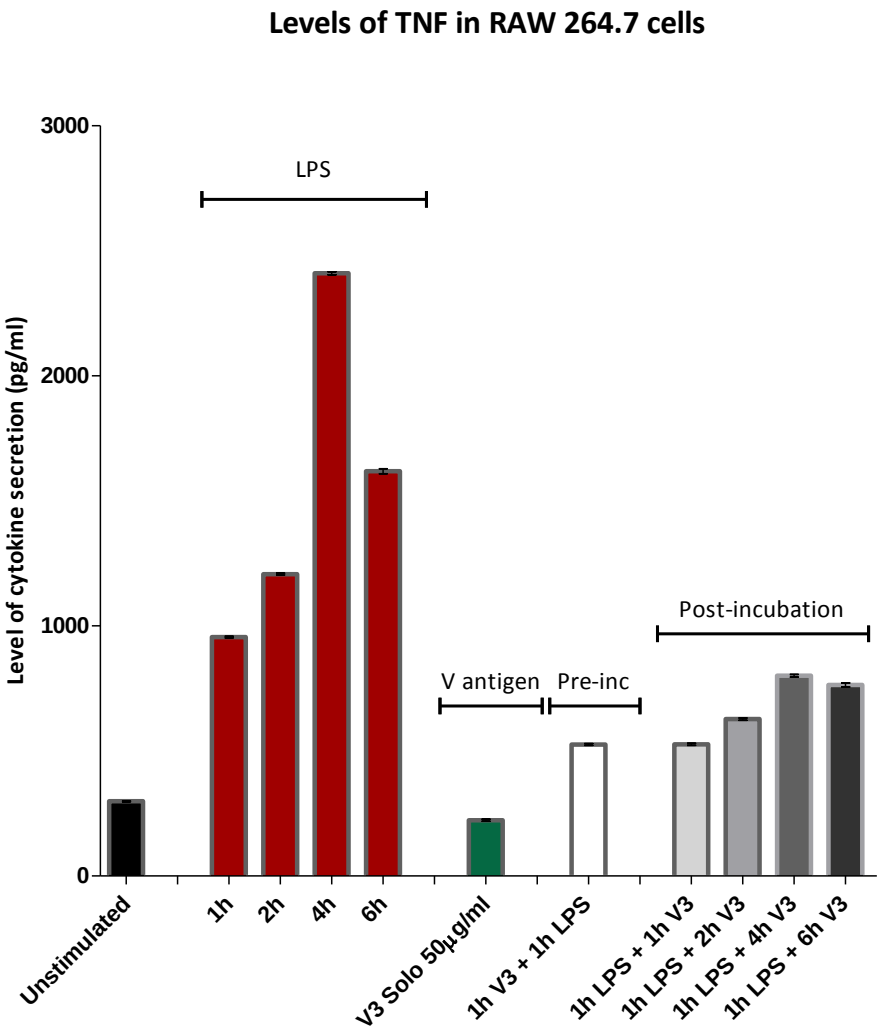


Figure 3.42: Secretion of TNF in RAW 264.7 cells in response to LPS, V3 and pre- or post-incubation with V3. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V3 for 1 hour; pre-incubated with 50µg/ml V3 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V3. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results demonstrated that LPS stimulated the release of IL-6. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V3 by itself did not stimulate TNF secretion. Our results showed a slight increase in TNF secretion when cells were pre- or post-incubated with V3 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.43).

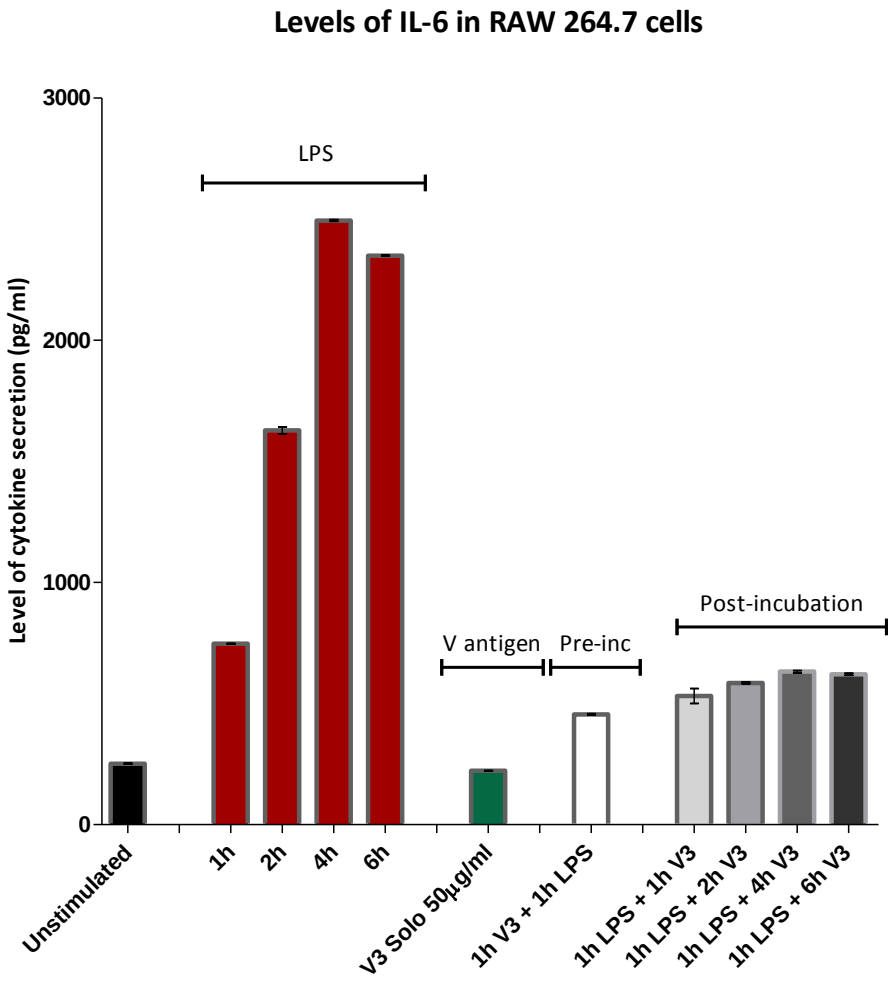


Figure 3.43: Secretion of IL-6 in RAW 264.7 cells in response to LPS, V3 and pre- or post-incubation with V3. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V3 for 1 hour; pre-incubated with 50µg/ml V3 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V3. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As predicted, LPS stimulated the release of IL-1β in RAW 264.7 cells. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V3 by itself did not stimulate IL-1β secretion. Our results showed a slight increase in TNF secretion when cells were pre- or post-incubated with V3 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.44).

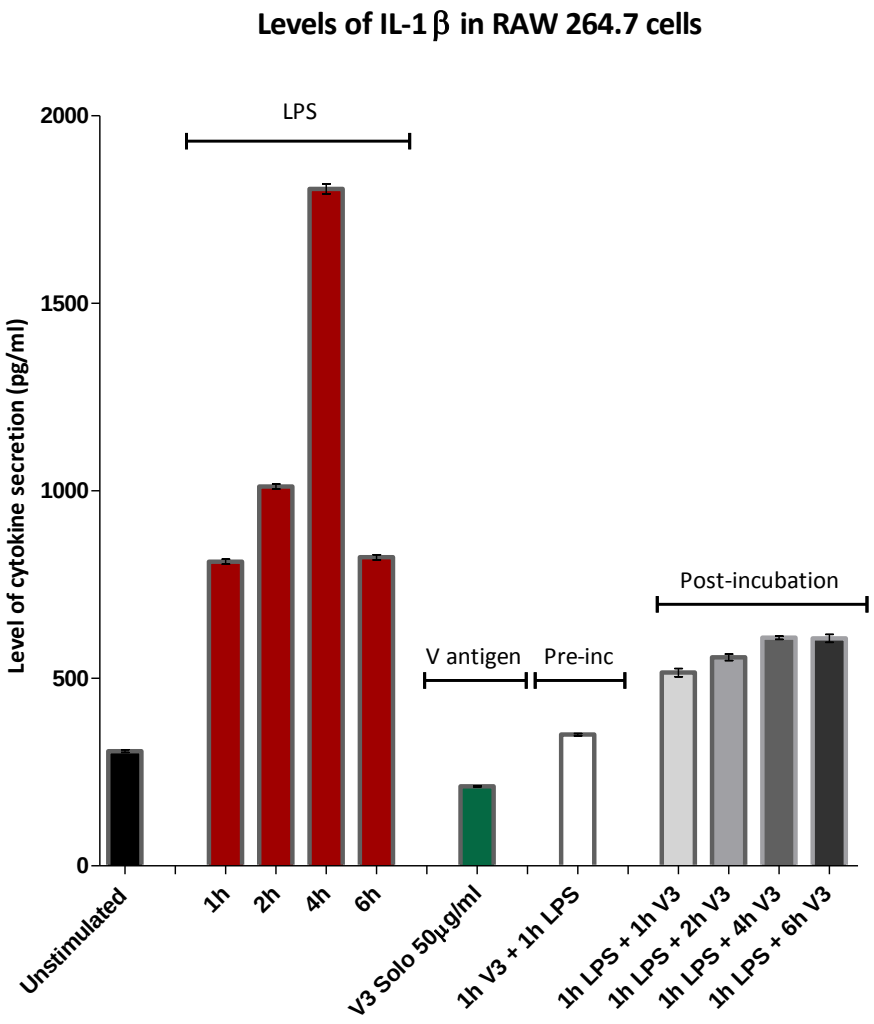


Figure 3.44: Secretion of IL-1β in RAW 264.7 cells in response to LPS, V3 and pre- or post-incubation with V3. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V3 for 1 hour; pre-incubated with 50µg/ml V3 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V3. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results showed that LPS stimulated the release of IL-10. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V3 by itself did not stimulate IL-10 secretion. Our results showed an increase in IL-10 secretion when cells were pre- or post-incubated for 1, 2 and 4 hours with V3 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS. When RAW 264.7 cells were post-incubated for 6 hours with V3 the levels of IL-10

increased to higher levels compared to cells stimulated with LPS with 1 hour (Figure 3.45).

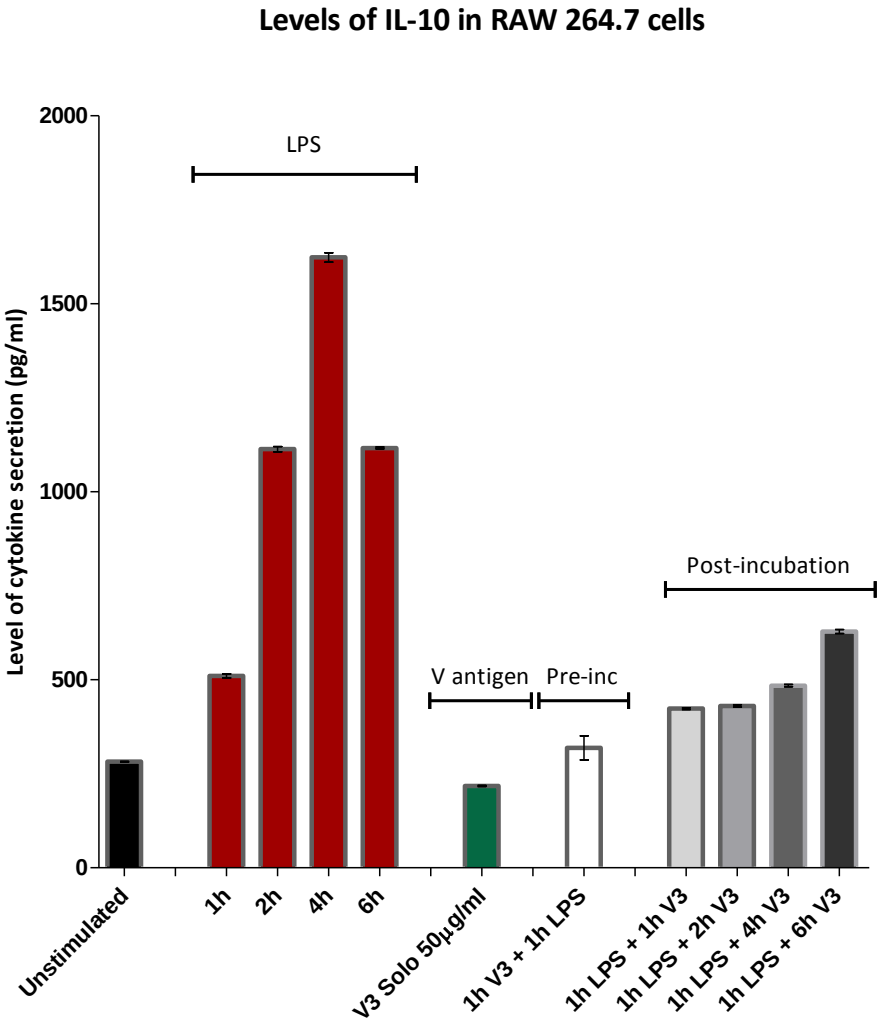


Figure 3.45: Secretion of IL-10 in RAW 264.7 cells in response to LPS, V3 and pre- or post-incubation with V3. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V3 for 1 hour; pre-incubated with 50µg/ml V3 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V3. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

3.4.3.3. Cytokine secretion in RAW 264.7 cells in response to V4

RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with 50µg/ml of the V1 for 1 hour. Additionally, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of the V1. All stimulations were carried out in 25cm^2 flasks. The supernatants were collected post stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

Out of the inflammatory cytokines analysed, only the release of TNF, IL-6, IL-1β and IL-10 were significantly increased in response to V4.

As foreseen, our results show that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V4 by itself did not stimulate TNF secretion. TNF secretion was significantly down-regulated in response to pre- and 1 hour post-incubation with V4 fraction, reaching as little as unstimulated levels. Our results showed a slight increase in TNF secretion when cells were post-incubated for 2, 4 and 6 hours with V4 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.46).

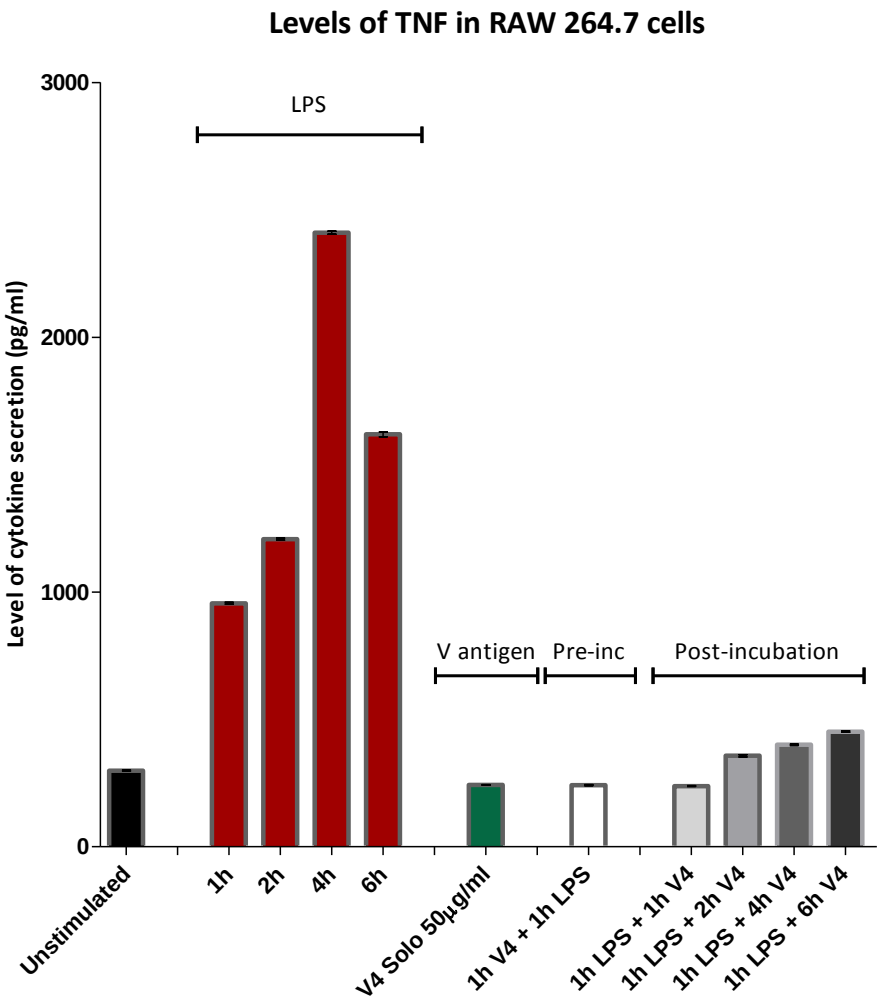


Figure 3.46: Secretion of TNF in RAW 264.7 cells in response to LPS, V4 and pre- or post-incubation with V4. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V4 for 1 hour; pre-incubated with 50µg/ml V4 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V4. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results show that LPS stimulated the release of IL-6 inflammatory cytokine. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V4 by itself did not stimulate IL-6 secretion. IL-6 secretion was significantly down-regulated in response to pre- and 1 hour post-incubation with V4 fraction, reaching as little as unstimulated levels. Our results showed a slight increase in IL-6 secretion when cells were post-incubated for 2, 4 and 6 hours with V4 compared to

unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.47).

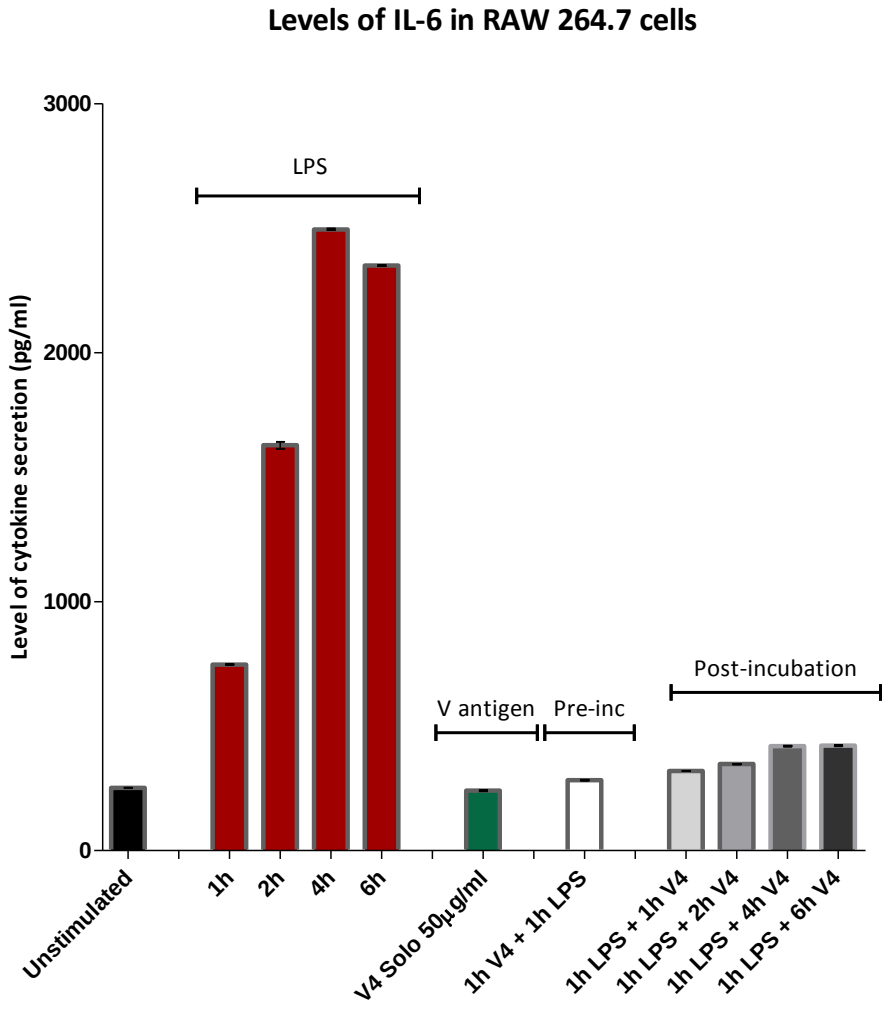


Figure 3.47: Secretion of IL-6 in RAW 264.7 cells in response to LPS, V4 and pre- or post-incubation with V4. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V4 for 1 hour; pre-incubated with 50µg/ml V4 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V4. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As anticipated, LPS resulted in the release of IL-1β inflammatory cytokine. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V4 by itself did not stimulate IL-1β secretion. IL-1β secretion was significantly down-

regulated in response to pre- and 1 hour post-incubation with V4 fraction, reaching as little as unstimulated levels. Our results showed a slight increase in IL-1β secretion when cells were post-incubated for 2, 4 and 6 hours with V4 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.48).

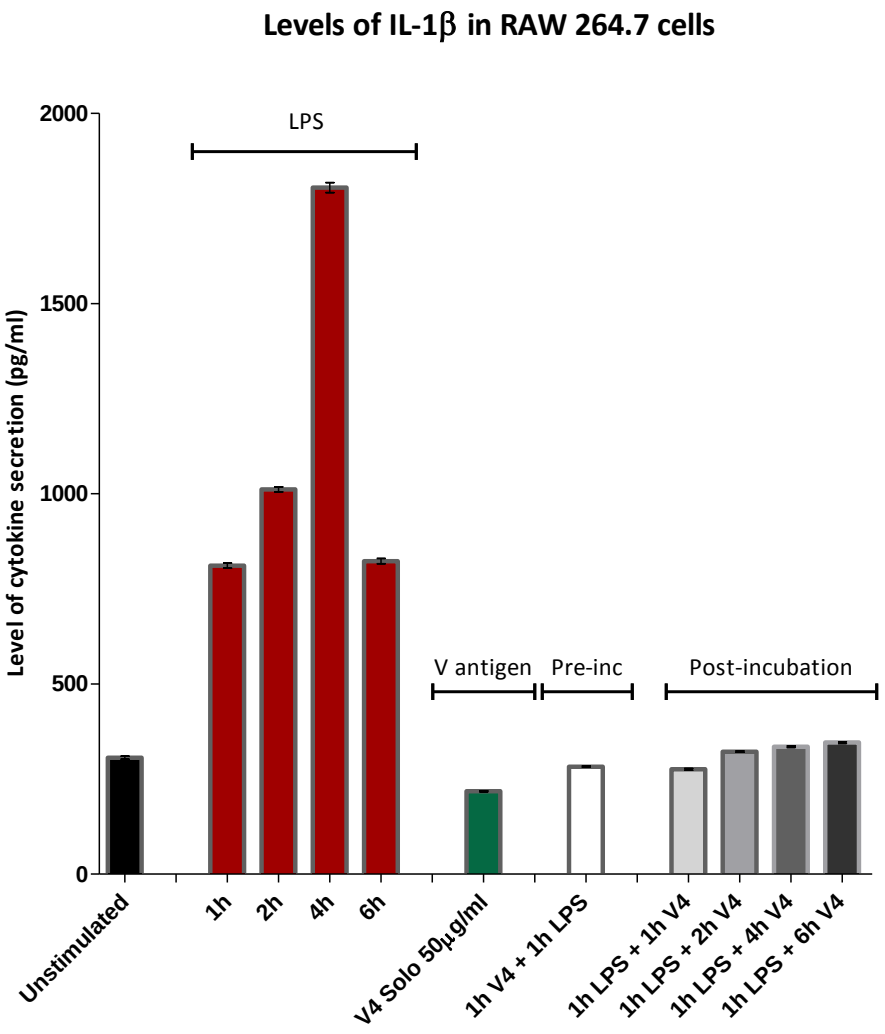


Figure 3.48: Secretion of IL-1β in RAW 264.7 cells in response to LPS, V4 and pre- or post-incubation with V4. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V4 for 1 hour; pre-incubated with 50µg/ml V4 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V4. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results show that LPS stimulated the release of IL-10 inflammatory cytokine. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V4 by itself did not stimulate IL-10 secretion. IL-10 secretion was significantly down-regulated in response to pre-, 1 and 2 hours post-incubation with V4 fraction, reaching as little as unstimulated levels. Our results showed a slight increase in IL-10 secretion when cells were post-incubated for 4 and 6 hours with V4 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.49).

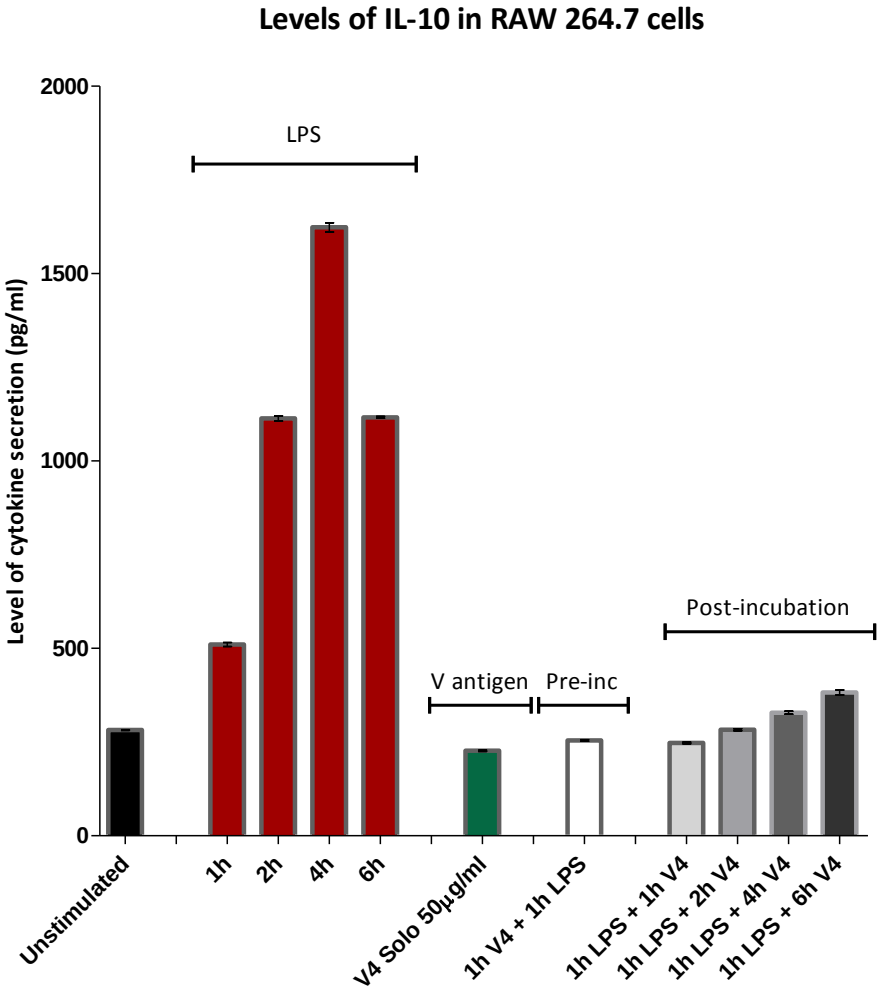


Figure 3.49: Secretion of IL-10 in RAW 264.7 cells in response to LPS, V4 and pre- or post-incubation with V4. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V4 for 1 hour; pre-incubated with 50µg/ml V4 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V4. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

3.4.3.4. Cytokine secretion in RAW 264.7 cells in response to V5

RAW 264.7 cells were stimulated for 1, 2, 4 and 6 hours with 100ng/ml of LPS as well as with 50µg/ml of the V1 for 1 hour. Additionally, RAW 264.7 cells were stimulated for 1 hour with 100ng/ml of LPS and either pre- or post-incubated for 1 hour or 1, 2, 4 and 6 hours, respectively, with 50µg/ml of the V1. All stimulations were carried out in 25cm^2 flasks. The supernatants were collected post stimulations for cytokine analysis using the Human Inflammation BD^{TM} Cytometric bead array kit (Section 2.8.2.2).

Out of the inflammatory cytokines analysed, only the release of TNF, IL-6, IL-1β and IL-10 were significantly increased in response to V5.

As expected, our results show that LPS stimulated the release of TNF. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V5 by itself did not stimulate TNF secretion. Our results showed a slight increase in TNF secretion when cells were pre- or post-incubated with V5 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.50).

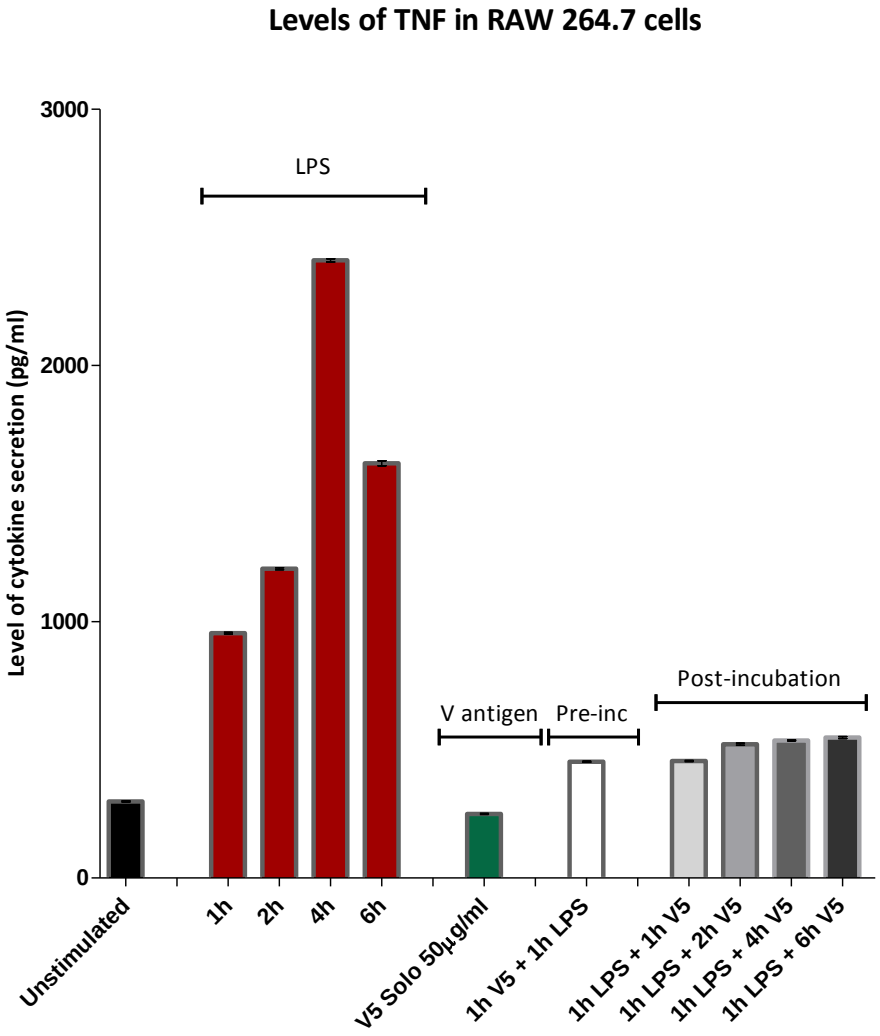


Figure 3.50: Secretion of TNF in RAW 264.7 cells in response to LPS, V5 and pre- or post-incubation with V5. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V5 for 1 hour; pre-incubated with 50µg/ml V5 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V5. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results show that LPS stimulated the release of Il-6. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V5 by itself did not stimulate IL-6 secretion. Our results showed a slight increase in IL-6 secretion when cells were pre- or post-incubated with V5 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.51).

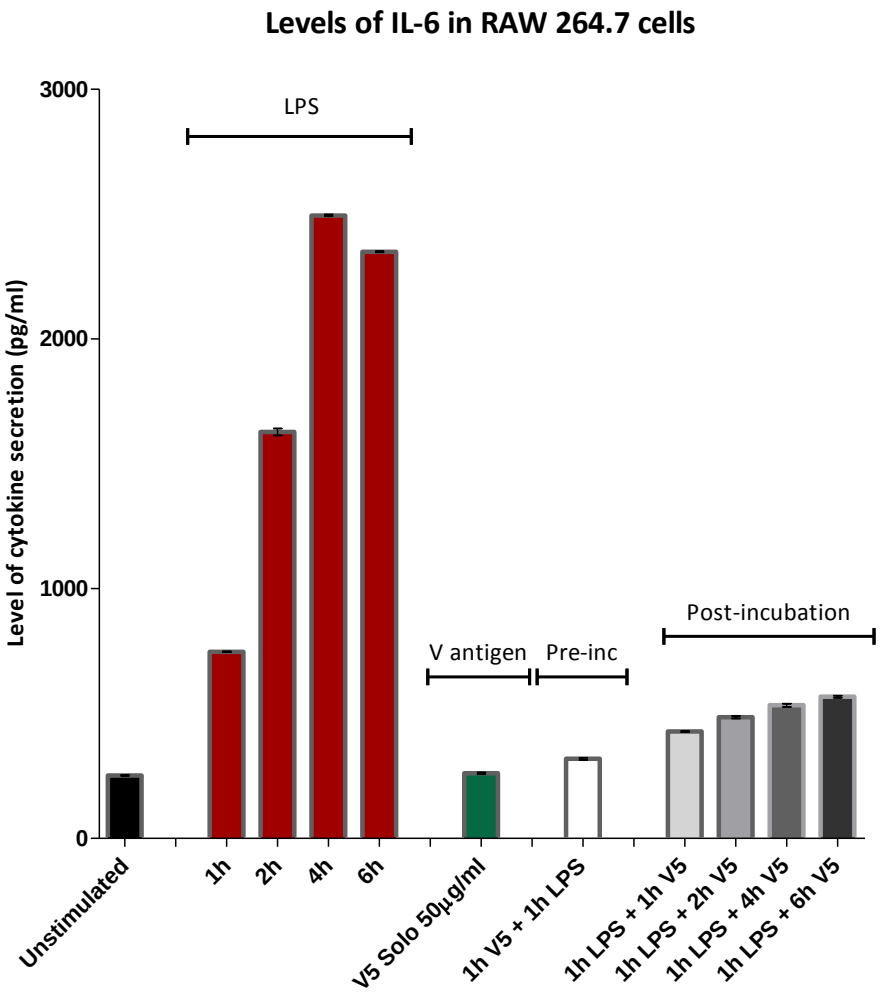


Figure 3.51: Secretion of IL-6 in RAW 264.7 cells in response to LPS, V5 and pre- or post-incubation with V5. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V5 for 1 hour; pre-incubated with 50µg/ml V5 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V5. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

As foreseen, LPS stimulated the release of IL-6 in RAW 264.7. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V5 by itself did not stimulate IL-1β secretion. Our results showed a slight increase in IL-1β secretion when cells were pre- or post-incubated with V5 compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.52).

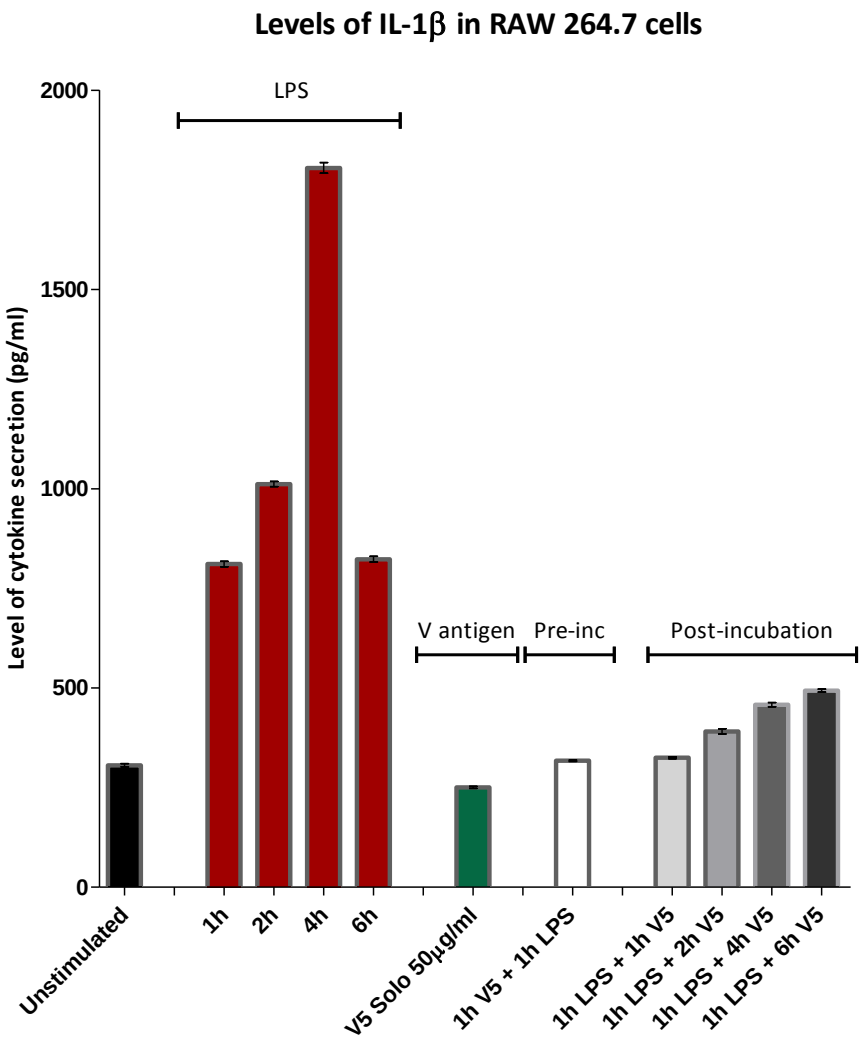


Figure 3.52: Secretion of IL-1β in RAW 264.7 cells in response to LPS, V5 and pre- or post-incubation with V5. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V5 for 1 hour; pre-incubated with 50µg/ml V5 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V5. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

Our results demonstrated that LPS stimulated the release of IL-10 in RAW 264.7. Its release increased over the first 4 hours and then lowered at the 6 hour mark. It was shown that V5 by itself did not stimulate IL-10 secretion. Pre-incubation with V5 did not alter the IL-10 secretion levels. Our results show that pos-incubating with V5 increased

IL-1β secretion compared to unstimulated cells, although with lower levels compared with cells stimulated with LPS (Figure 3.53).

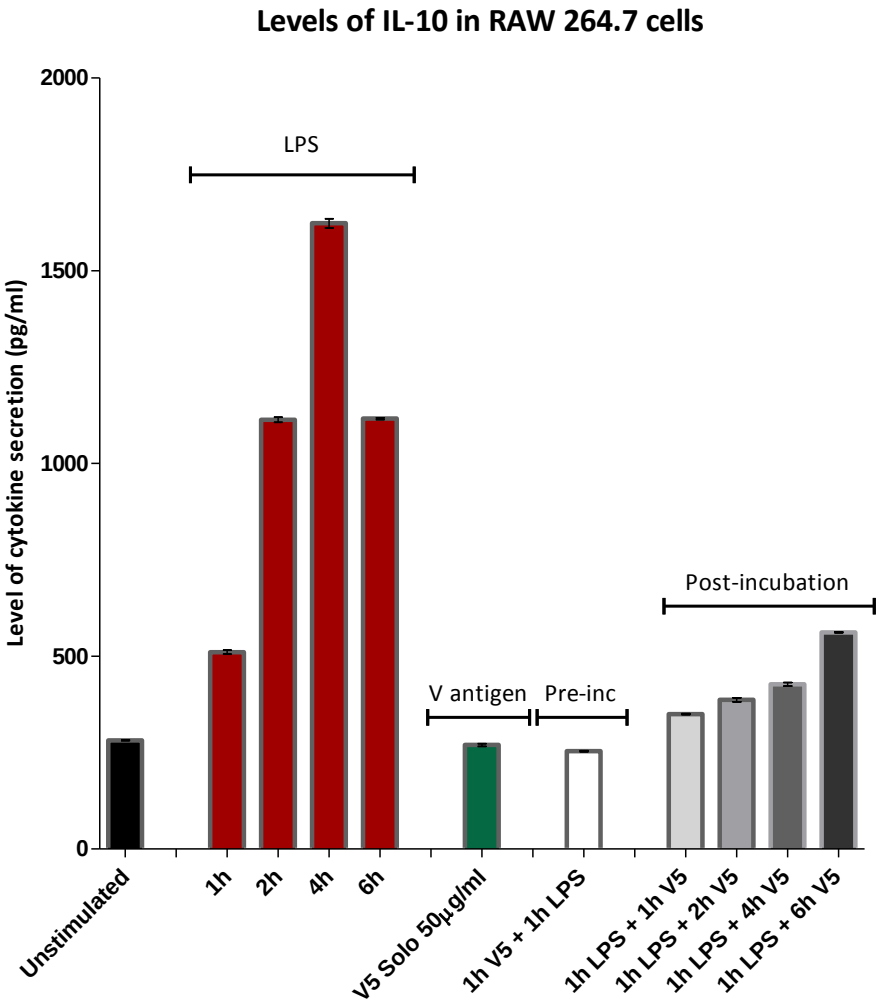


Figure 3.52: Secretion of IL-10 in RAW 264.7 cells in response to LPS, V5 and pre- or post-incubation with V5. RAW 264.7 cells were stimulated with: 100ng/ml LPS for 1, 2, 4 and 6 hours; 50µg/ml of V5 for 1 hour; pre-incubated with 50µg/ml V5 for 1 hour and stimulated with 100ng/ml of LPS for 1 hour; or stimulated with 100ng/ml LPS for 1 hour and post-incubated for 1, 2, 4 and 6 hours with 50µg/ml V5. The cytokines were measured in the cell supernatant using a flow cytometric cytokine bead array system (Becton Dickinson). Unstimulated controls were performed. The data is statistically significant ($p<0.05$). The data represents the mean of three independent experiments.

3.4.4. Conclusion

Previous studies have shown that, not only the entire, but also small regions of *Yersinia pestis* V antigen can provide protection against the plague. It has been suggested that *Y. pestis* V antigen contains a number of protective fragments that are located in a central region between amino acids (a.a.) 135 and 275 (Hill *et al.*, 1997). Vernazza *et al.* (2009) aimed to identify the minimum protective fraction of *Y. pestis* V antigen that could provide protection against plague. In order to do so, they generated small regions of the V antigen, residing between the central a.a. 135-275 fragment.

In order to investigate the functional domain of V antigen that was responsible for the immunomodulatory effects that we observed, V antigen fractions had to be produced. V antigen fractions were cloned in *E. coli*, cultured in Luria broth and the protein isolated using affinity chromatography. In this study, out of the six fragments that possibly could confer protection, proposed by Vernazza *et al.* (2009), four were chosen – V1, V3, V4 and V5. V1 was selected because it has been previously shown to be protective against plague, V3 due to a N-terminal deletion whereas V4 and V5 a C-terminal deletion type.

Initially, we tested if RAW 264.7 cells when stimulated with LPS would activate NF-κB and caspase-1. Our results showed that both cell types do activate both NF-κB and caspase-1.

Once it was established that LPS was able to trigger NF-κB signalling cascade and caspase-1 p-10, whether the different V antigen fractions were able to modulate this response was investigated. Our results revealed in RAW 264.7 cells V1, V4 and V5 inhibited NF-κB and caspase-1 activation over time. V3 fraction was able to decrease NF-κB and caspase-1 activation, but not to the same extent as the other fractions.

The next step was to evaluate the inflammatory cytokine release and if the different fractions had an effect in the levels secreted by RAW 264.7 cells. Out of the

inflammatory cytokines analysed (IL-8, IL-1β,IL-6, IL-10, TNF and IL-12p70), only the release of IL-1β, IL-6, IL-10 and TNF were significantly increased in response to LPS. Our results show V1, V3, V4 and V5 can inhibit the production of TNF, IL-6, IL-1β and IL-10 inflammatory cytokines. The highest reduction of cytokine release was seen on V1 followed by V4, V5 and last V3, therefore the functional domain of V antigen responsible for the effects that we have observed lies within the amino acids 135-275.

3.5. Intracellular trafficking and targeting of exogenous V antigen

The innate immune system has an essential role in recognising a range of invading pathogens and is responsible to mount an inflammatory response in order to remove them in a fast and efficient manner. It employs germ-lined encoded receptors, termed pattern recognition receptors (PRRs), which are able to recognise molecular "signatures" that exist on pathogens. These PRRs are expressed on immune cells and are able to distinguish a great variety of microbial ligands, including ones from Gram-negative and positive bacteria. A tight regulation of the innate immune system is key to keep the body alive. The loss of regulation or overstimulation of innate immunity can lead to detrimental and often-fatal symptoms. Septic shock is a prime example of a condition that reflects this overreaction of the hosts innate immune system, where bacterial components cause the over secretion of cytokines, which trigger a chain of damaging events that cause this condition. This study concerned with the effects of V antigen has in the innate immune system. It is becoming apparent that the presence of V antigen may have the effect of altering cellular response to LPS.

Our data has shown that V antigen interferes with PRRs, ultimately leading to a modified cellular response. The next step in this study was to use confocal microscopy to check whether V antigen from *Y. pestis* associates with PRRs, signalling molecules and cellular organelles.

3.5.1. Intracellular effect of V antigen on TLR4 and MyD88

To investigate the effect of V antigen on TLR4 and MyD88, RAW 264.7 cells were grown on 8 well glass slides (Section 2.12.2) and indirect immunofluorescence (Section 2.7.2) was used to label RAW 264.7 cells. Slides were viewed using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue) (Section 2.12.3.1). RAW cells were stimulated in eight time-points: unstimulated (0 hour); 1 hour

LPS, 1 hour V antigen; and 1 hour LPS followed by either 1, 2, 4 or 6 hour(s) of V antigen. The cells were subsequently fixed and labelled for TO-PRO (blue (nuclear stain)), TLR4 (green (FITC)) and MyD88 (red (Cy3)) (Figure 3.53).

In unstimulated RAW 264.7 cells, TLR4 and MyD88 did not to co-localise. On the other hand, upon LPS stimulation, TLR4 did co-localise with MyD88. Similarly to unstimulated, when RAW 264.7 cells were incubated for 1 hour with V antigen TLR4 and MyD88 did not co-localise.

When RAW 264.7 cells were post-incubated with V antigen, TLR4 and MyD88 did co-localise in the first two post-incubation time-points. In contrast, when RAW 264.7 cells were post-incubated for 4 and 6 hours, TLR4 and MyD88 did not associate. An interesting result occurred at the 2 hour post-incubation mark, where MyD88 seems to cluster.

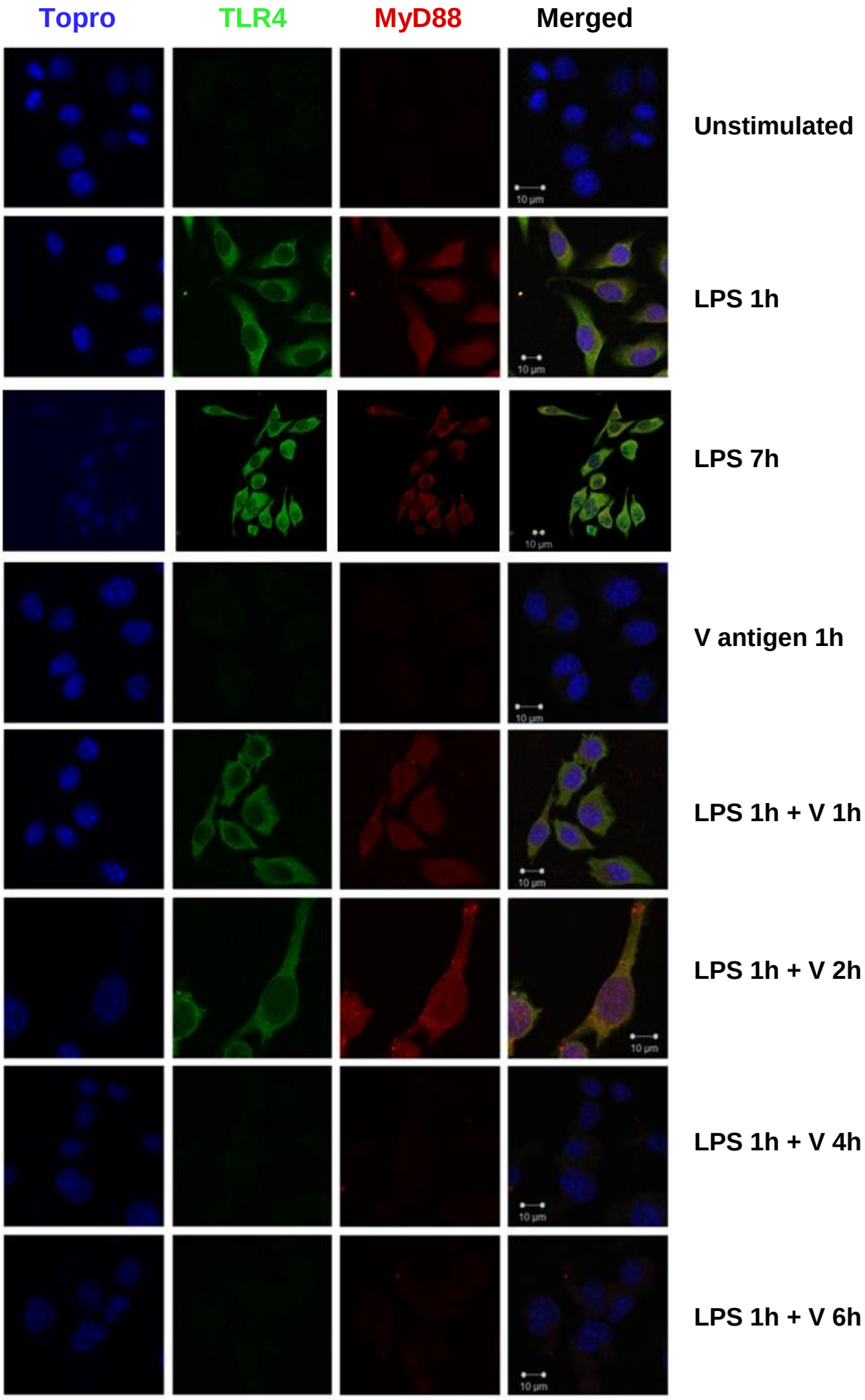

Topro
TLR4
MyD88
Merged
Unstimulated
LPS 1h
LPS 7h
V antigen 1h
LPS 1h + V 1h
LPS 1h + V 2h
LPS 1h + V 4h
LPS 1h + V 6h
10 µm

Figure 3.53: Intracellular effect of V antigen on TLR4 (green (FITC)) and MyD88 (red (Cy3)) in RAW 264.7 cells. TO-PRO (blue) was used to stain the nucleus. RAW 264.7 were either unstimulated or stimulated for 1 and 7 hour with 100ng/ml of LPS; incubated for 1 hour with 50µg/ml of V antigen; or stimulated for 1 hour with 100ng/ml of LPS followed by a post-incubation with 50µg/ml of V antigen for 1, 2, 4 or 6 hour(s). The cells were subsequently fixed and labelled via indirect immunofluorescence. The images are representative from a number of independent experiments. The cells were imaged using a Carl Zeiss LSM 510 META confocal microscope, used in conjunction with Zeiss LSM software. Scale bar, 10µm.

3.5.2. V antigen co-localisation with TLR4 and MyD88 over time

In order to determine if V antigen associates with TLR4 or MyD88, RAW 264.7 cells were grown on 8 well glass slides (Section 2.12.2) and indirect immunofluorescence (Section 2.7.2) was used to label RAW 264.7 cells. Cells were incubated with V antigen conjugated to Oregon Green (V-OG) for 10 minutes, 30 minutes, 1 hour and 2 hours (Section 2.12.1). Following stimulation with V-OG the cells were fixed and labelled for TLR4 and MyD88 with a specific antibody and the appropriate secondary antibody conjugated with Cy5 (blue) and Cy3 (red), respectively (Figure 3.54). Slides were viewed using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue) (Section 2.12.3.1).

The first thing that can be said about the results obtained is that V antigen is internalised within 10 minutes. Also at this time point it seems that V antigen is co-localising with MyD88. It also seems to associate with TLR4 but not as much as it does with MyD88. Similarly, V antigen seems to co-localise with MyD88 and, to a lesser extent with TLR4 throughout the remaining incubation time-points.

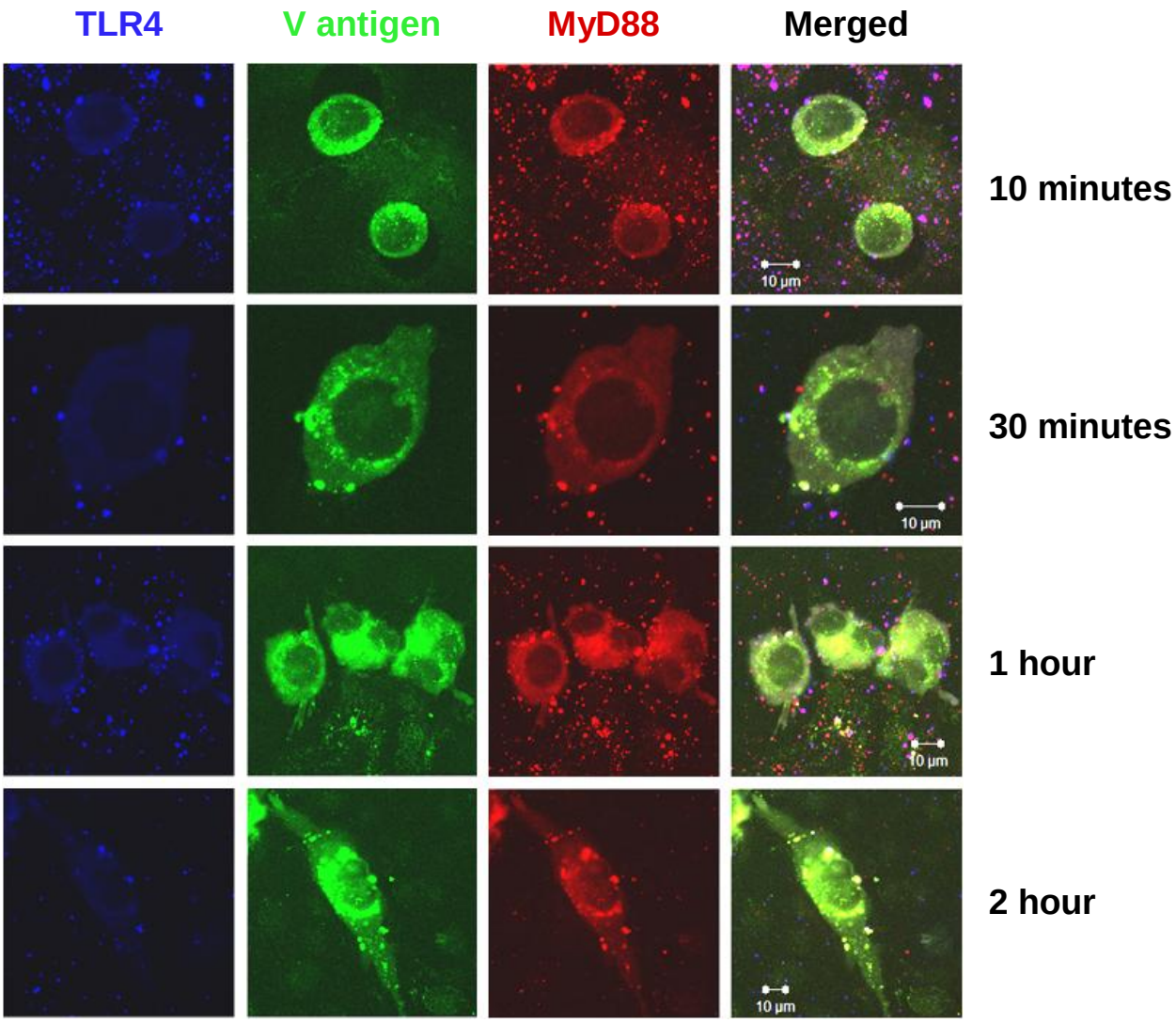


Figure 3.54: V antigen co-localisation with TLR4 and MyD88. RAW 264.7 were incubated with V antigen conjugated with Oregon green (V-OG) for 10 minutes, 30 minutes, 1 hour and 2 hours. The cells were subsequently fixed and labelled via indirect immunofluorescence for TLR4 (blue (Cy5)) and MyD88 (red (Cy3)). The cells were imaged using a Carl Zeiss LSM 510 META confocal microscope, used in conjunction with Zeiss LSM software. The images are representative from a number of independent experiments. Scale bar, 10µm.

3.5.3. V antigen co-localisation with caspase-1 and NLRP12 over time

In order to determine if V antigen associates with caspase-1 or NLRP12, RAW 264.7 cells were grown on 8 well glass slides (Section 2.12.2) and indirect immunofluorescence (Section 2.7.2) was used to label RAW 264.7 cells. Cells were incubated with V antigen conjugated to Oregon Green (V-OG) for 10 minutes, 30 minutes, 1 hour and 2 hours (Section 2.12.1). Following stimulation with V-OG the cells were fixed and labelled for caspase-1 p20 and NLRP12 with a specific antibody and the appropriate secondary antibody conjugated with and Cy5 (blue) and Cy3 (red), respectively (Figure 3.55). Slides were viewed using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue) (Section 2.12.3.1).

Confocal microscopy revealed that that V antigen is internalised within 10 minutes. At this time point it seems that V antigen does not associate with either NLRP12 or caspase-1. V antigen only seems to start co-localising with NLRP12 after 30 minutes of incubation. The subsequent incubation time-points show that V antigen is strongly co-localising with NLRP12. On the contrary, V antigen does not seem to associate with caspase-1 through the incubation time-points.

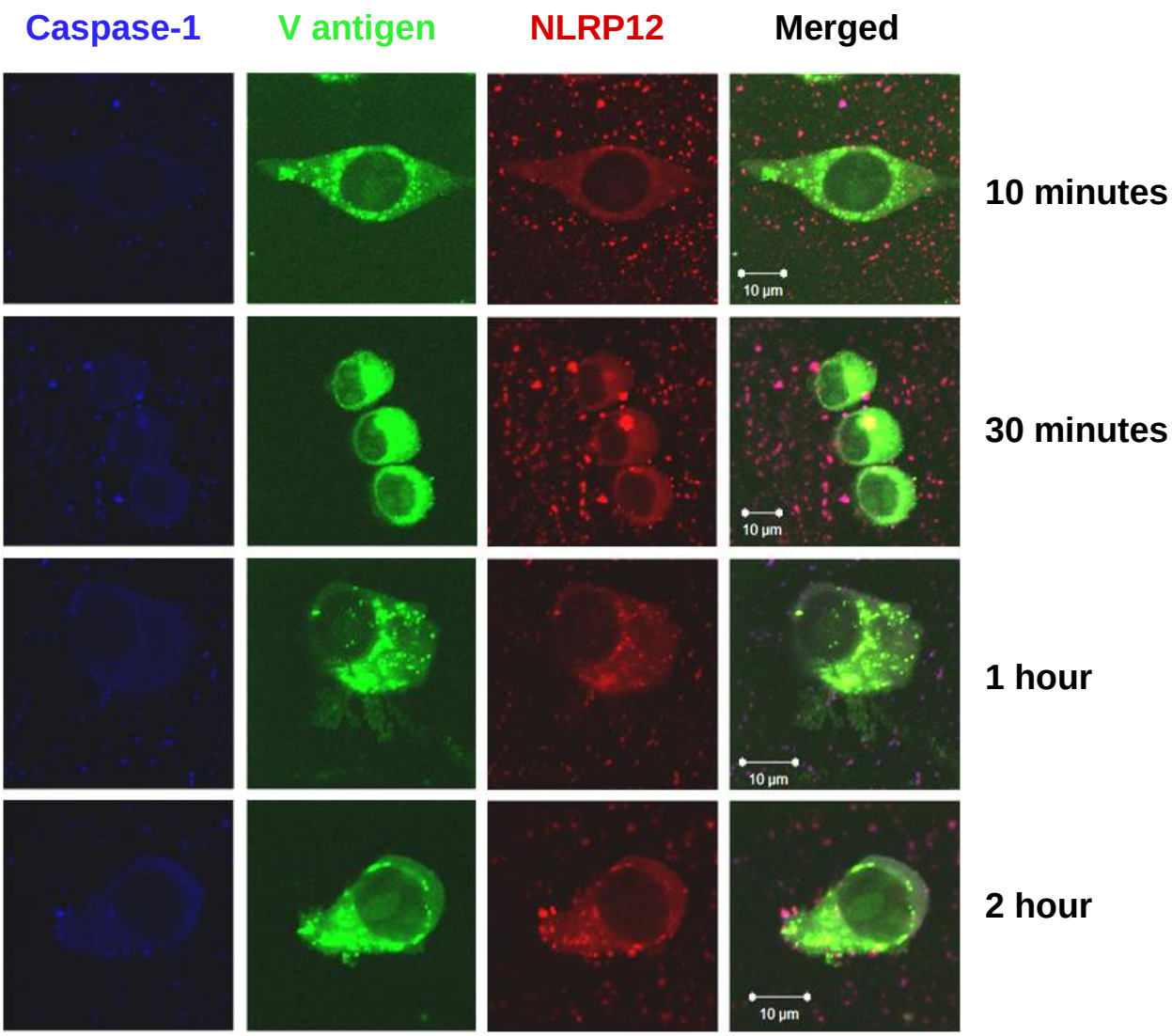


Figure 3.55: V antigen co-localisation with caspase-1 and NLRP12. RAW 264.7 were incubated with V antigen conjugated with Oregon green (V-OG) for 10 minutes, 30 minutes, 1 hour and 2 hours. The cells were subsequently fixed and labelled via indirect immunofluorescence for caspase-1 p20 (blue (Cy5)) and NLRP12 (red (Cy3)). The cells were imaged using a Carl Zeiss LSM 510 META confocal microscope, used in conjunction with Zeiss LSM software. The images are representative from a number of independent experiments. Scale bar, 10µm.

3.5.4. Intracellular co-localisation of V antigen with IRF-7 and IRF3

In this study we also utilised RAW 264.7 cells to see whether V antigen would associate IFN regulatory factor 7 (IRF7) and IRF3. Both these regulatory factors are part of the MyD88-independent pathway, which translocate to the nucleous and initiate transcription of IFN and IFN-inducible genes in order to induce type-I IFN expression.

In order to determine if V antigen associates with IRF7 and IRF3, RAW 264.7 cells were grown on 8 well glass slides (Section 2.12.2) and indirect immunofluorescence (Section 2.7.2) was used to label RAW 264.7 cells. Cells were incubated with V antigen conjugated to FITC for 10 minutes (Section 2.12.1). Following stimulation with V-FITC the cells were fixed and labelled for IRF7 and IRF3 with a specific antibody and the appropriate secondary antibody conjugated with and Cy5 (blue) and Alexa fluor 633 (red), respectively (Figure 3.56). Slides were viewed using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue) (Section 2.12.3.1).

Confocal microscopy revealed that that V antigen strongly associates with both IRF7 and IRF3. This shows that V antigen might not only be modulating the MyD88-dependent but also the MyD88-independent pathway.

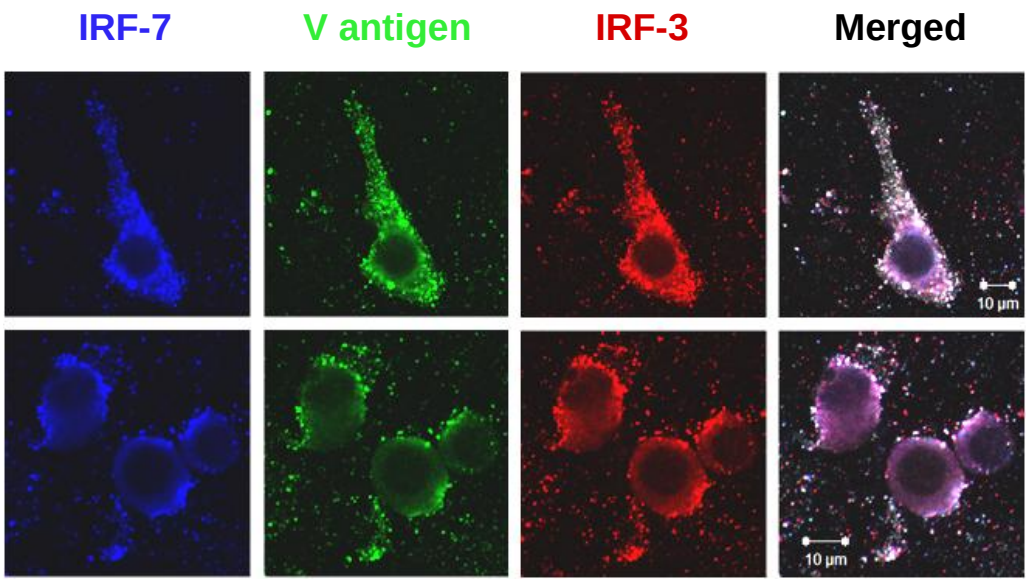


Figure 3.56: V antigen co-localisation with IRF-7 and IRF-3. RAW 264.7 were incubated with V antigen conjugated FITC (V-FITC) for 10 minutes. The cells were subsequently fixed and labelled via indirect immunofluorescence for IRF-7 (blue (Cy5)) and IRF-3 (red (Alexa 633)). The cells were imaged using a Carl Zeiss LSM 510 META confocal microscope, used in conjunction with Zeiss LSM software. The images are representative from a number of independent experiments. Scale bar, 10µm.

3.5.5. Intracellular co-localisation of V antigen with EEA1 and NLRP3

In order to determine whether V antigen was internalised and where it was being targeted to within the cell, confocal microscopy was utilised.

RAW 264.7 were incubated with V antigen conjugated to FITC (V-FITC) for 10 minutes, 30 minutes, 1 hour and 2 hours. Following stimulation with V-FITC the cells were fixed and the endosomes were labelled with an antibody against EEA1, a protein within the early endosomes, and the appropriate secondary antibody conjugated with Cy5 (blue) (Figure 3.57). In addition, NLRP3 was labelled with a specific antibody and the appropriate secondary antibody conjugated to Cy3 (red). Slides were viewed using a Zeiss LSM 510 META confocal microscope under a 63x / 1.4 Oil DIC objective, utilising the three fluorescent wavelengths of 488 (Argon – green), 543 (red) and 633 (blue) (Section 2.12.3.1).

It was shown that when RAW 264.7 cells were incubated with V antigen, within minutes, it associated with EEA1, showing that V antigen might be concentrated in endosomes. Similarly, V antigen was shown that it does co-localise with NLRP3. The remaining incubation time-points show that V antigen co-localises with both EEA1 and NLRP3.

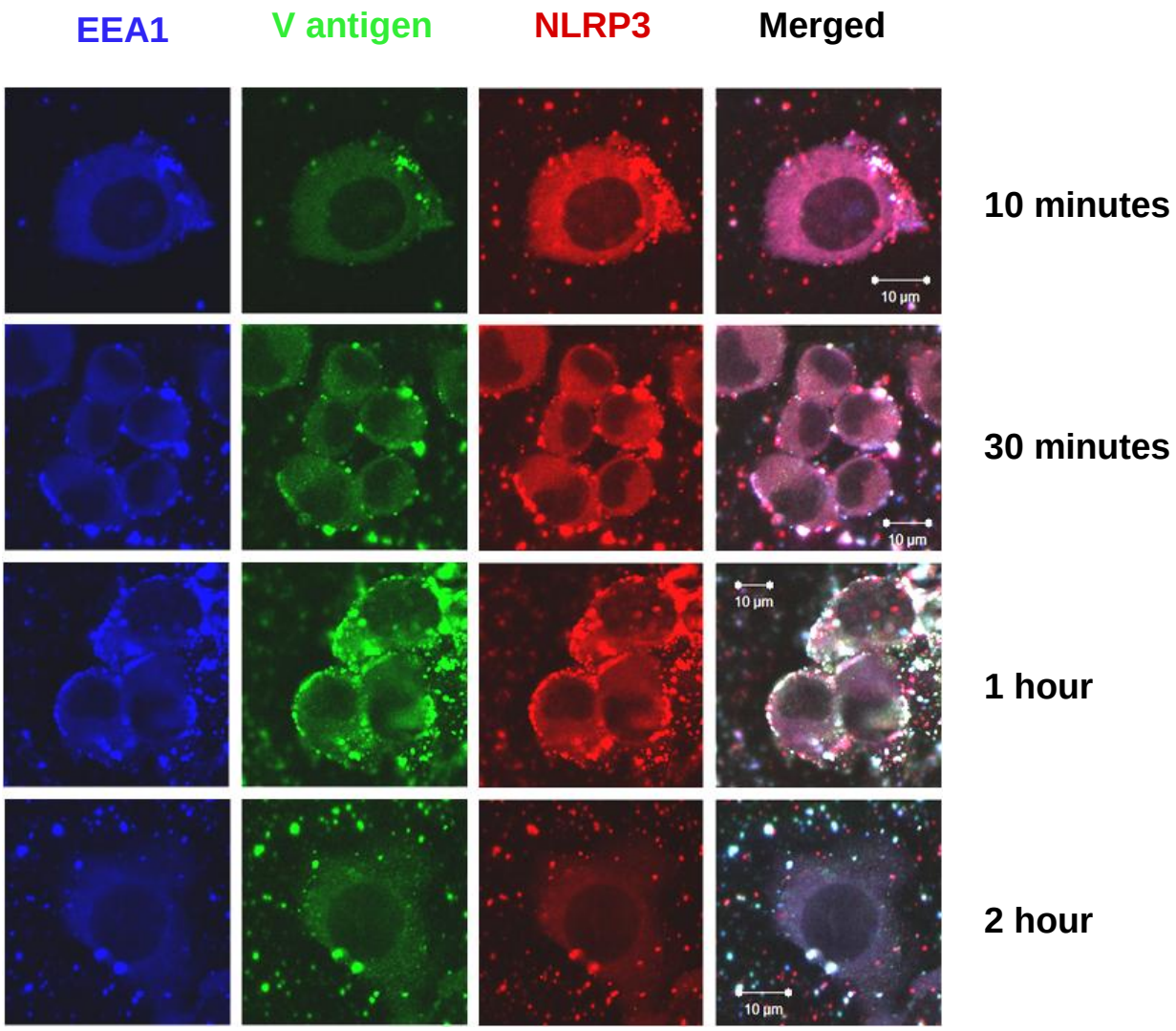


Figure 3.57: V antigen co-localisation with EEA1 and NLRP3. RAW 264.7 were incubated with V antigen conjugated FITC (V-FITC) for 10 minutes, 30 minutes, 1 hour and 2 hours. The cells were subsequently fixed and labelled via indirect immunofluorescence for EEA1 (blue (Cy5)) and NLRP3 (red (Cy3)). The cells were imaged using a Carl Zeiss LSM 510 META confocal microscope, used in conjunction with Zeiss LSM software. The images are representative from a number of independent experiments. Scale bar, 10µm.

3.5.6. Conclusion

V antigen has been shown to associate with TLRs and to modulate their signalling, ultimately altering the cytokine secretion. Confocal microscopy was used in order to test the effect of V antigen on TLR4 and MyD88 in response to LPS. It was shown that unstimulated cells and cells incubated for 1 hour with V antigen did not activate TLR4 or MyD88. On the other hand, when RAW 264.7 cells were stimulated with LPS, both TLR4 and MyD88 showed, as expected, activation and co-localisation. Interestingly, when cells were stimulated with LPS and post-incubated with V antigen our results show that the first two post-incubation periods there is still activation and co-localisation of both TLR4 and MyD88, but by the 4 and 6 post-incubation time-point, TLR4 and MyD88 co-localisation seems to be inhibited.

Our results show that there is an influx of V antigen right from the 10 minute mark.

Additionally, confocal microscopy was also used in order to test V antigen colocalisation with TLR4, NLRP3 and NLRP12 over a 2 hour period. It seems that V antigen is co-localising with NLRP3 and NLRP12 straight from within 10 minutes of stimulation and throughout the 2 hours. Similarly, but to a lesser extent, V antigen does co-localise with TLR4.

Signalling molecules, such as MyD88, IRF3 and IRF7 were also tested to elucidate whether or not there is an association with V antigen. Our results show that both MyD88 and IRFs co-localised with V antigen, demonstrating that not only V antigen interacts with the dependent (MyD88) but also independent (IRF3 and IRF7) pathways. Caspase-1 also seems to interact with V antigen, but not excessively.

Finally, cells were incubated with V antigen and labelled for EEA1, a protein within the early endosomes. Our results show that V antigen associates with EEA1, which shows that it might be concentrated within endosomes.

3.6. *Yersinia pestis* V antigen as a therapeutic intervention for sepsis

Sepsis is the leading cause for all hospitalised patients and the leading cause in death in non-coronary intense care units (ICUs). It also remains an important cause of childhood morbidity and mortality (Alberti et al., 2003, Martin et al., 2003). Despite the availability of potent antibiotics to which the bacteria are highly sensitive, the overall mortality rate is higher than 30%.

It is now widely accepted that the over-reaction of the host occurs at the level of the innate immune system and is directly linked to the recognition of bacterial cell wall components, such as lipopolysaccharide (LPS) from Gram-negative bacteria. On entry to the bloodstream, bacteria and their products (principally LPS) trigger an intense inflammatory process which results in a loss of regulation of the innate immune system. This loss of regulation or excessive stimulation of innate immunity can lead to a dangerous and often fatal symptoms. Septic shock is the purest example of this deregulation, where an overreaction of the host innate immune system lead to harmful symptoms. The search to pinpoint molecules that are able to modulate the innate immune responses is decisive in the successful development of a therapeutic intervention for sepsis.

As already mentioned, currently therapy is limited to anti-infectives and supportive care. These approaches only give the body time to recover, but do not treat the cause of the problem. Our group has been working in trying to identify possible inhibitors of the “sensing apparatus” in order to dampen the host’s inflammatory response. In this study we tested the effects of the virulence factor from a bacterium, *Yersinia pestis* V antigen (LcrV) on LPS-induced responses. V antigen is a multifunctional protein that has been shown to have immunomodulatory features (Derewenda et al., 2004, Fields et al., 1999, Leary et al., 1995, Pettersson et al., 1999). *Y. pestis*, the causative agent of the three main plague pandemics has been responsible for over 200 million deaths.

The bacterium has been so successful because it encodes several virulence factors, one of which is V antigen. In this part of the study, we investigated whether this protein was able to inhibit LPS responses *in vivo* using a mouse model of sepsis.

3.6.1. Determination of LPS concentration needed to induce septic shock

In order to determine the optimum concentration of LPS that is needed to induce septic shock, male CD-1 out-bred mice (6-8 weeks) were randomly grouped (10 mice per group) and injected by intraperitoneal (i.p.) (Section 2.13.2) injection with different concentrations (50, 60, 70 and 80 mg/Kg) of *E. coli* LPS using a sterile 1ml syringe (BD Plastipak^{TM}) with a 25 GA1 0.5x25mm needle (BD Microlance^{TM} 3). Since our mouse model of sepsis takes place over 72 hours, the suitability of each LPS concentration was determined by its ability to induce septic shock and death with 24 hours from LPS administration.

LPS-induced death was observed for LPS of 60, 70 and 80mg/Kg. When 50mg/Kg was administered 100% survival at 24 hours was recorded. Our data showed that 60mg/Kg of LPS was sufficient in order to induce septic shock and death by 24 hours (Figure 3.58), thus it was decided to use this concentration in all future experiments.

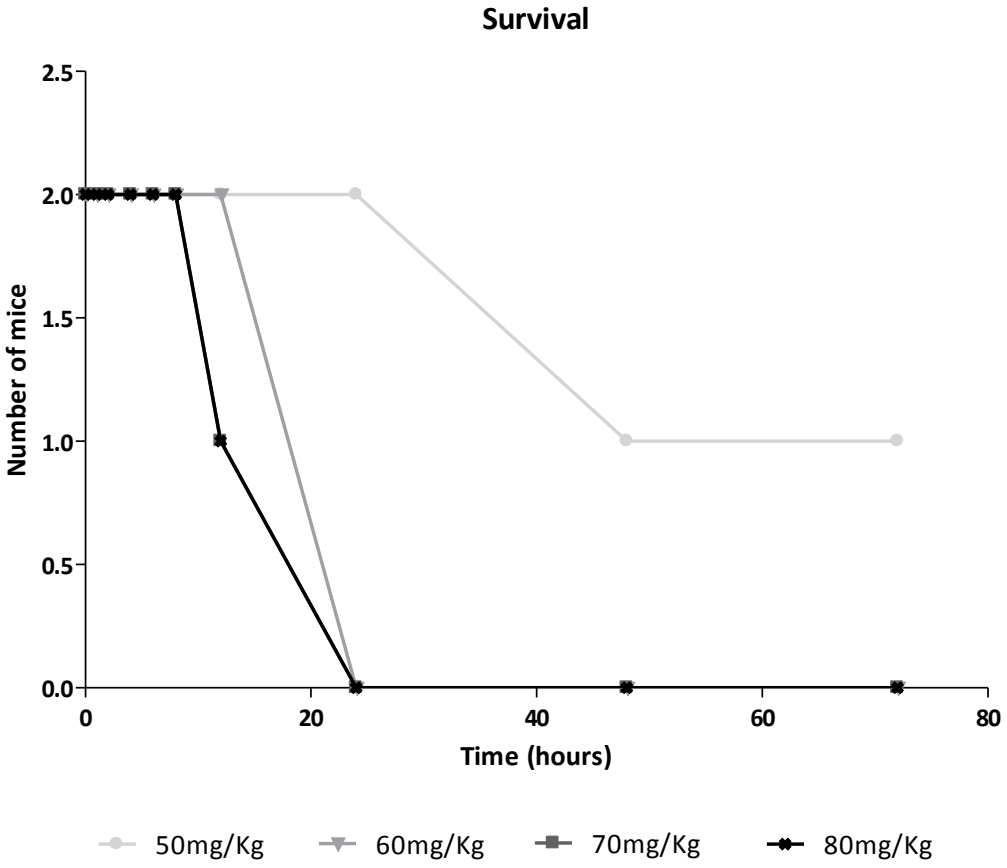


Figure 3.58: Determination of LPS concentration needed to induce septic shock. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected intraperitoneal (i.p.) injection with different concentrations (50, 60, 70, 80mg/Kg) of *E. coli* LPS. Mortality was determined over a 72 period. Data points are from three independent experiments.

3.6.2. Determination of optimal V antigen concentration

In order to determine the optimum concentration of V antigen that is needed to protect mice from LPS-induced septic shock, male CD-1 out-bred mice (6-8 weeks) were randomly grouped (10 mice per group) and injected by intraperitoneal (i.p.) (Section 2.13.2) injection with different concentrations (500µg/Kg and 1mg/Kg) of V antigen 1 hour after LPS administration. A sterile 1ml syringe (BD Plastipak^{TM}) with a 25 GA1 0.5x25mm needle (BD Microlance^{TM} 3) was used. Since our mouse model of sepsis takes place over 72 hours, the suitability of each V antigen concentration was

determined by its ability to protect mice from septic shock and death with 24 hours from LPS administration.

Protection from LPS-induced death was observed for V antigen of 500µg/Kg. Our data showed that 500µg/Kg of V antigen was sufficient in order to protect mice from LPS-induced septic shock and death by 24 hours (Figure 3.59), thus it was decided to use this concentration in all future experiments.

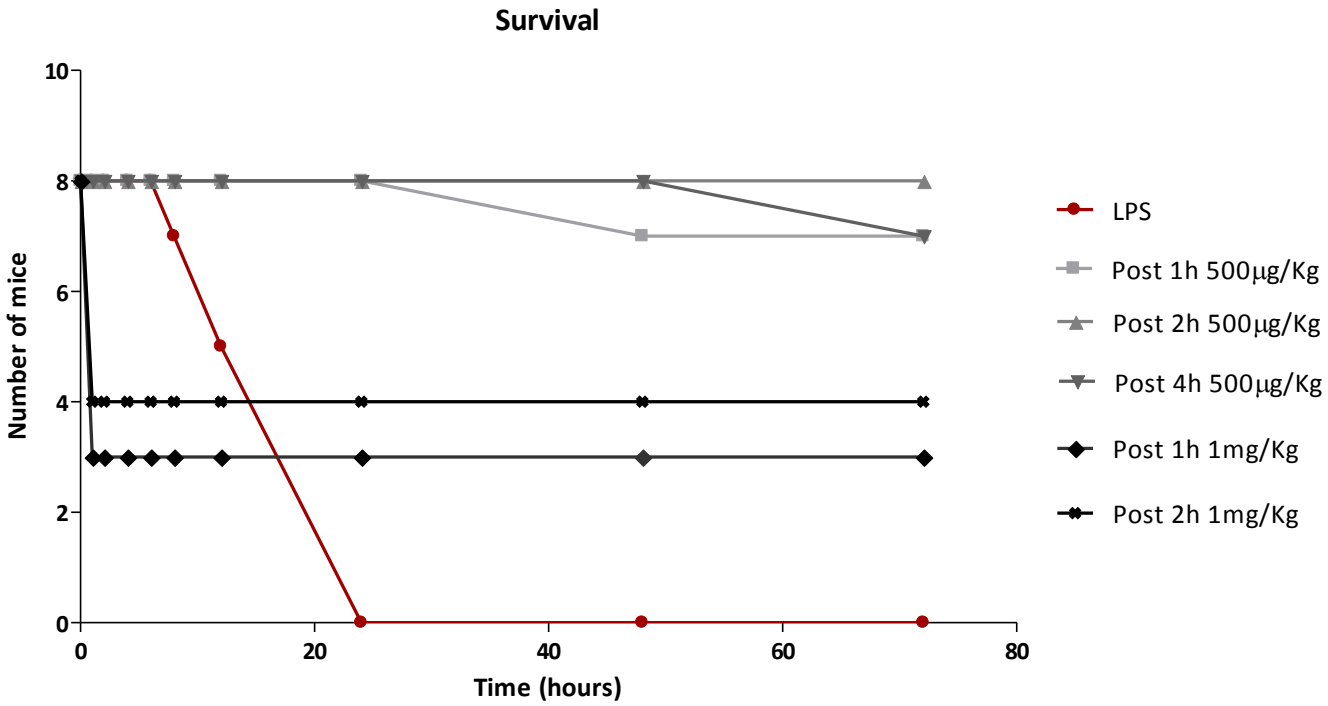


Figure 3.59: Determination of V antigen concentration needed to protect mice from LPS-induced septic shock. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected intraperitoneal (i.p.) injection with different concentrations (500µg/Kg and 1mg/Kg) of V antigen 1 hour after LPS stimualtion. Mortality was determined over a 72 period. Data points are from three independent experiments.

3.6.3. Pre-treatment with V antigen improves survival in response to LPS

In order to determine whether V antigen is able to protect the mice if they are pre-treated with V antigen prior to LPS administration, *in vivo* experiments were employed. For these studies, male CD-1 out-bred mice (6-8 weeks) were randomly grouped (10 mice per group) and injected by intraperitoneal (i.p.) (Section 2.13.2) injection with different 60mg/Kg of *E. coli* LPS, which was previously shown to be sufficient to induce shock within 24 hours. Improved survival of animals and inhibition of cytokines secretion was used in order to determine efficacy. It was shown that V antigen was able to improve survival up to 72.5% when administered 1 hour to LPS administration (Figure 3.60).

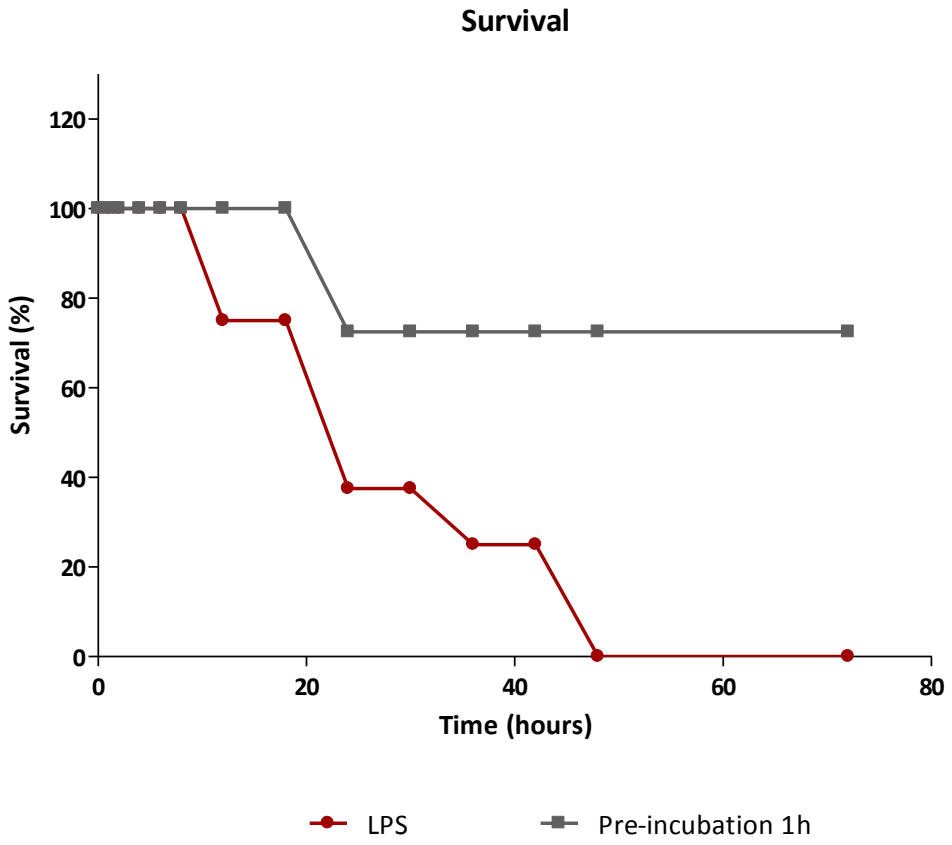
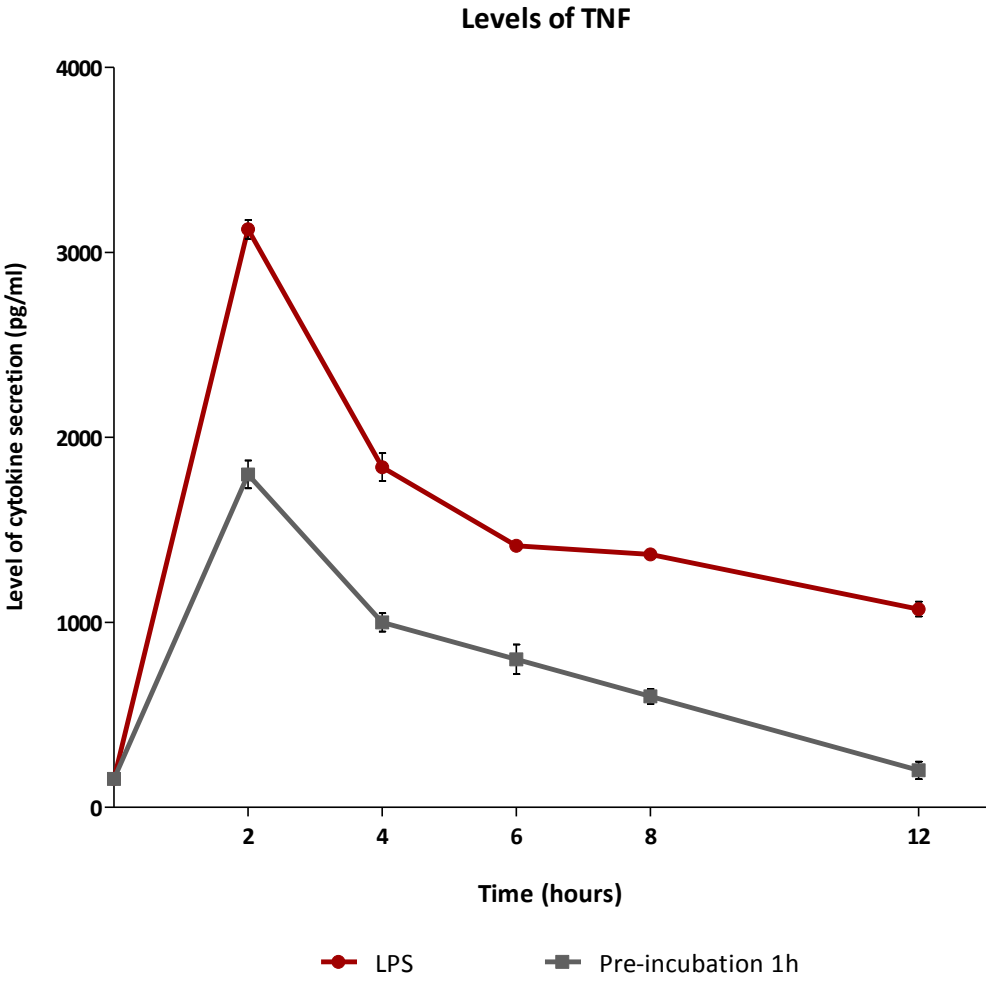


Figure 3.60: Pre-treatment with V antigen improves survival in response to LPS. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected via intraperitoneal

(i.p.) injection with V antigen 0.5mg/Kg 1 hour prior to 60mg/Kg *E. coli* LPS administration. Survival was determined over a 72 period. Data points are from three independent experiments.

3.6.4. Pre-treatment with V antigen inhibits inflammatory responses *in vivo*

Blood (250µl) was collected at time-points 0, 2, 4, 6, 8 and 12 hours from the tail vain of the mice. Inflammatory cytokine levels, of TNF and IL-1β, in the serum were determined using a cytokine bead array (CBA) (Becton Dickinson). It was shown that LPS induced an inflammatory response, reaching its highest at 2 hours following LPS administration (Figure 3.61). Pre-treatment of mice with 0.5mg/Kg V antigen 1 hour prior to the LPS administration revealed that it almost halved the amount of TNF (Figure 3.61, top) as well as IL-1β (Figure 61, bottom).



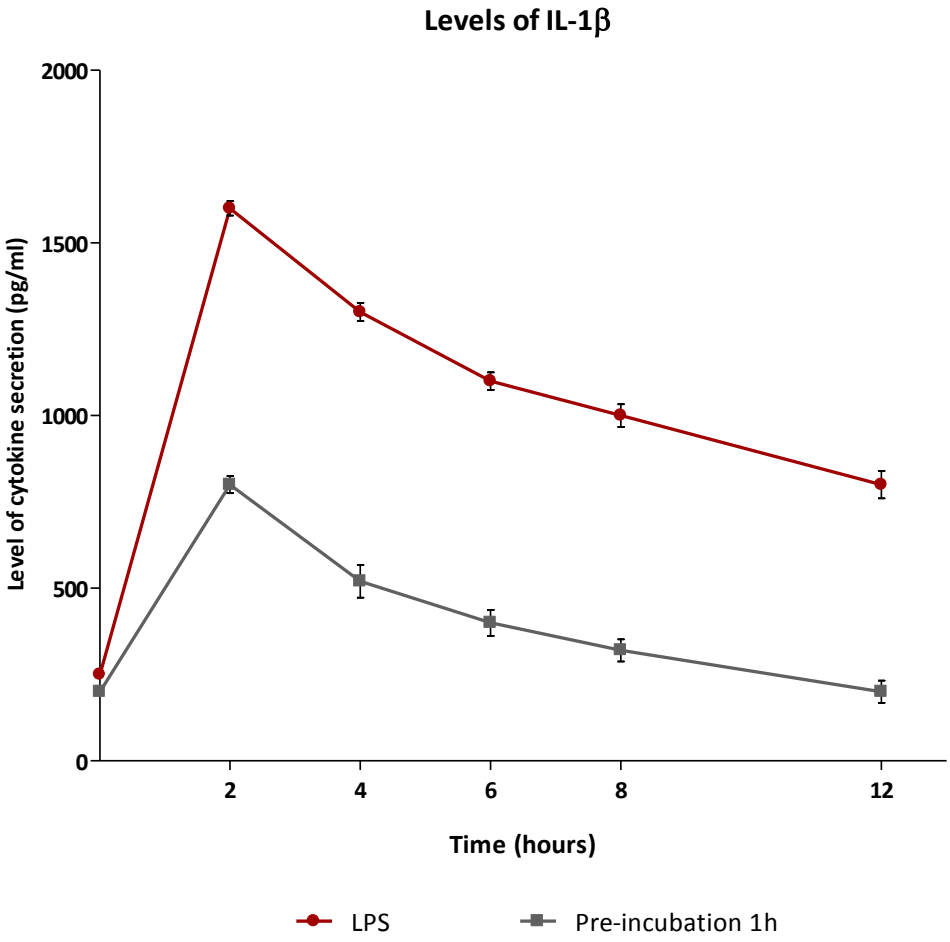


Figure 3.61: Analysis of inflammatory parameters during fatal sepsis. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected intraperitoneal (i.p.) injection with V antigen 0.5mg/Kg 1 hour prior to 60mg/Kg *E. coli* LPS administration. 250µl of tail vein blood were taken from CD-1 mice at 0, 2, 4, 6, 8 and 12 hours. The level of TNF (top) and IL-1β (bottom) in the serum was determined using a cytokine bead array (CBA) system obtained from Becton Dickinson. Data points are from three independent experiments.

3.6.5. Post-treatment with V antigen improves survival in response to LPS

If V antigen is to be used as a therapeutic intervention, it would be administered after the bacterial infection has presented itself. Therefore, in order to determine whether V antigen is able to protect the mice following LPS administration *in vivo* experiments were employed. For these studies, male CD-1 out-bred mice (6-8 weeks) were randomly grouped (10 mice per group) and injected by intraperitoneal (i.p.) (Section 2.13.2) injection with different 60mg/Kg of *E. coli* LPS, which was previously shown to be sufficient to induce shock within 24 hours. V antigen (0.5 mg/Kg) was administered by i.p. injection at different time points (1, 2, 4 and 6 hours) following LPS administration. Improved survival of animals and inhibition of cytokine secretion was used in order to determine efficacy. It was shown that V antigen was able to improve survival up to 80% even when treatment was postponed for up to 6 hours (Figure 3.62).

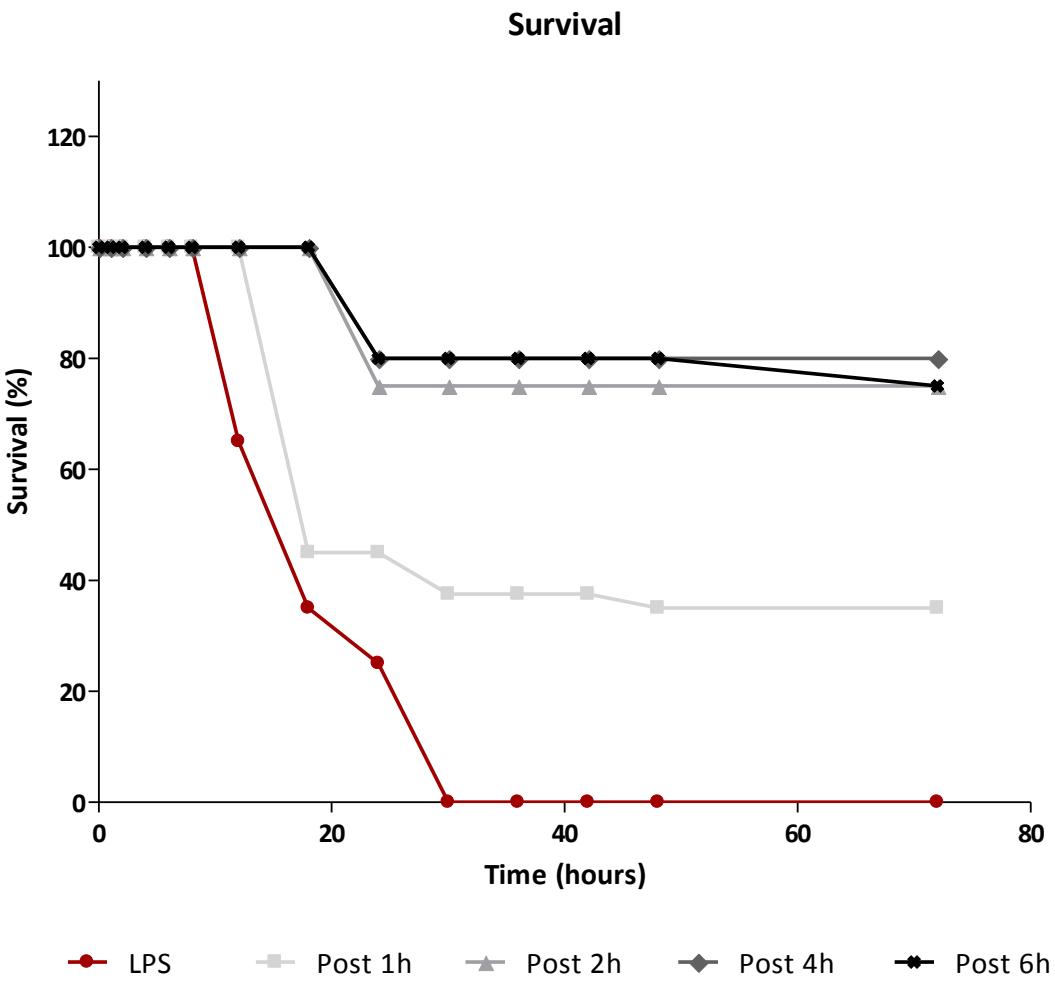
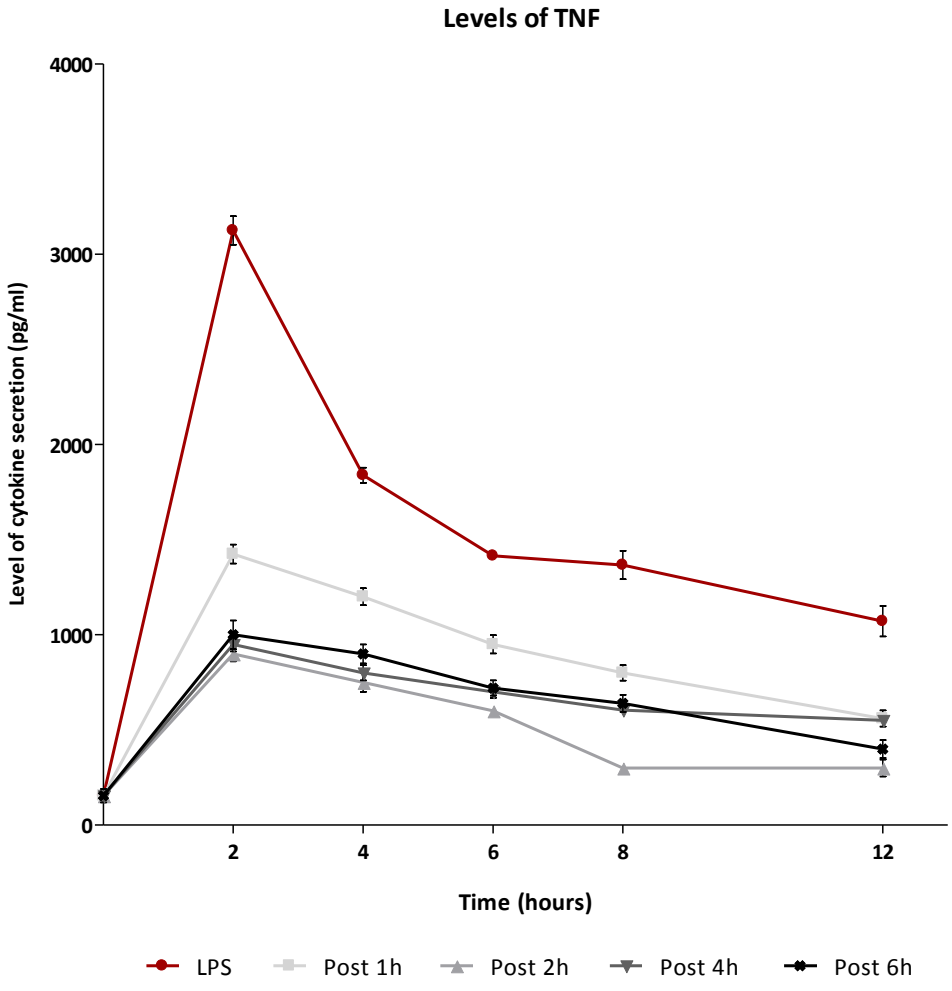


Figure 3.62: Post-treatment with V antigen improves survival in response to LPS. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected via intraperitoneal (i.p.) injection with V antigen 0.5mg/Kg at different time points (1, 2, 4 and 6 hours) following 60mg/Kg *E. coli* LPS administration. Survival was determined over a 72 period. Data points are from three independent experiments.

3.6.6. Post-treatment with V antigen inhibits inflammatory responses *in vivo*

In order for V antigen to be used therapeutically for the treatment of sepsis, it has to show efficacy when administered after LPS treatment. In order to test the efficacy of LcrV in treating sepsis following LPS administration, Blood (250µl) was collected at time-points 0, 2, 4, 6, 8 and 12 hours from the tail vain of the mice. Inflammatory cytokine levels, of TNF and IL-1β, in the serum were determined using a cytokine bead array (CBA) (Becton Dickinson). It was shown V antigen was able to able to inhibit inflammatory responses even if the mice were treated 6 hours following LPS treatment (Figure 3.63). In particular it was shown to be more effective when administered 4-6 hours after LPS treatment, where the TNF (Figure 3.63, top) and IL-1β (Figure 3.63, bottom) levels were reduced by almost 80% when compared to the mice treated with LPS alone.



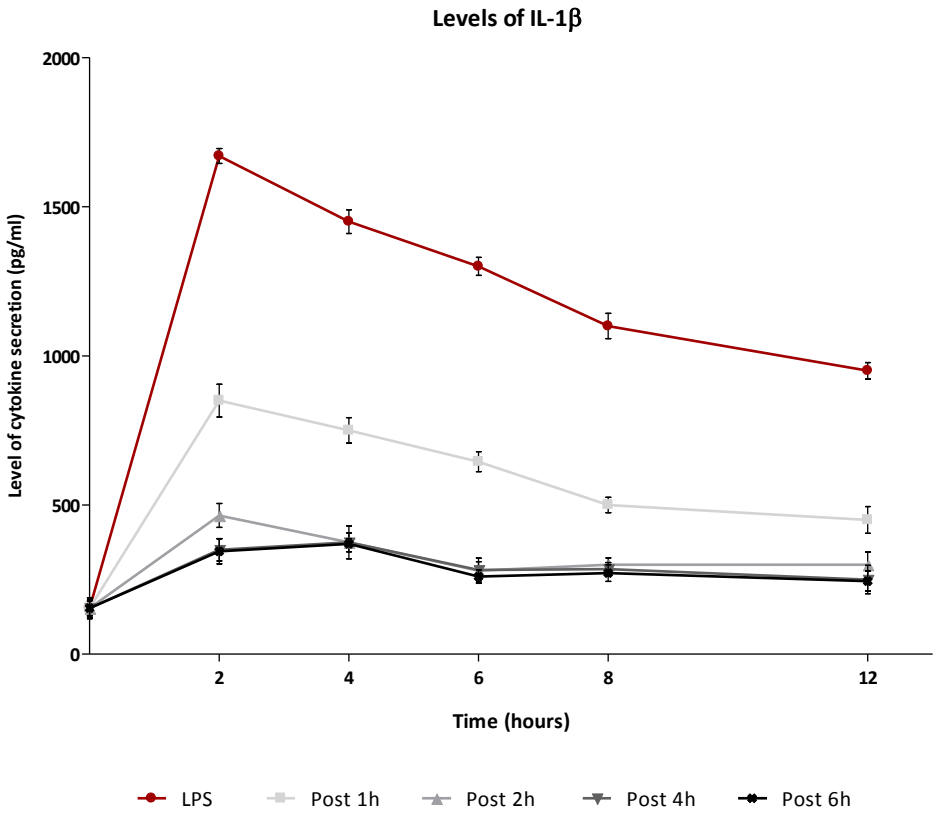


Figure 3.63: Analysis of inflammatory parameters during fatal sepsis. Male CD-1 mice (6-8 weeks) were randomly grouped (10 mice per group) and injected intraperitoneal (i.p.) injection with V antigen 0.5mg/Kg 1 hour following to 60mg/Kg *E. coli* LPS administration. 250µl of tail vein blood were taken from CD-1 mice at 0, 2, 4, 6, 8 and 12 hours. The level of TNF (top) and IL-1β (bottom) in the serum was determined using a cytokine bead array (CBA) system obtained from Becton Dickinson. Data points are from three independent experiments.

3.6.7. Conclusion

Sepsis is an area of unmet clinical need and thus there are very few new products that are reaching the market. At the moment there is no treatment for the disease, except antibiotics, which can kill the bacterium but do not neutralise its LPS and the only new product that has been licensed in the last few years in order to reduce mortality in severe sepsis is recombinant activated Protein C or Xigris – which targets the coagulation cascade and has since been withdrawn from the market for having low efficacy as well as multiple side-effects.

In this study we decided to test the efficacy of V antigen treatment both as a prophylactic intervention as well as a therapeutic treatment for sepsis. We have utilized a mouse model of sepsis where we induce death by LPS. When we tested V antigen's prophylactic properties by pre-incubating the mice with 0.5 mg/animal we found that V antigen could improve survival by 70% and reduce pro-inflammatory cytokines TNF-a and IL-1β by almost half the amount.

Most importantly when we tested V antigen's therapeutic potential, we treated animals at 2, 4, 6 and 8 hour post administration of LPS. V antigen was shown to dramatically increase the survival of the mice to 80% even when they were treated 6 hours post infection. Interestingly it was shown that the longer the treatment was postponed the better, as the 4 and 6 h post-treatment gave us the best survival rates.

As expected the pro-inflammatory response was also significantly decreased, as shown by the levels of TNF and IL-1β observed.

Chapter 4

Discussion

Sepsis is a clinical disorder that is an often-fatal systemic inflammatory response to bacterial infection. This medical condition is paradoxical, seeing as the system that has evolved to protect the host against microbial infection is fundamentally responsible for injury and damage to the host. Bacterial products, like lipopolysaccharide (LPS), which are recognised by the host lead to an oversecretion of inflammatory cytokines that have a detrimental effect to the host.

Patients with severe sepsis and septic shock manifest striking heterogeneity including age, underlying diseases, diverse microbial etiology, local infection site and genetic makeup. This problem remains a major hurdle which makes the discovery of new therapies for severe sepsis and septic shock especially difficult. Over the past 4 decades, the outcome for septic patients has improved significantly, and yet this improvement is almost solely due to advances in the supportive care given to critically ill patients and the use of antimicrobial agents (Suffredini and Munford, 2011). Over the years, great effort and expenditure has gone in order to develop new treatments to reverse the harmful and often terminal effects seen on septic patients. With the exception of a short-lived drug, no new treatments have been developed.

Sepsis is not only the leading cause of death in non-coronary intensive care units (ICUs) but also one of the most common causes of morbidity and mortality for all hospitalised patients (Alberti *et al.*, 2003, Martin *et al.*, 2003). Seen that there is no current therapy for sepsis and this condition has a mortality rate higher than 30% and is responsible for more than 750,000 and 30,000 annual cases in USA and England, Wales and Northern Ireland, respectively, it is crucial to identify molecules that are able to modulate the host response to bacterial infection (Angus et al., 2001, Harrison et al., 2006, Martin et al., 2003, Padkin et al., 2003).

Yersinia pestis, the aetiological agent of the plague, is a bacterium responsible for hundreds of millions of deaths throughout history. It invades the mammalian body,

often through a flea bite, and proceeds to create a fulminating disease that, more often than not, tends to kill the host within a matter of days. It is so successful because *Y. pestis* possesses several plasmids that encode virulence factors that modulate the host immune system. V antigen, also known as LcrV, is one of these virulence factors and it is encoded by the *lcrV* gene in the pCD1/pYV plasmid. This 326-residue, 37kDa soluble protein has many functions, from being critical for gene regulation within the bacterium and for translocating other virulence factors (Yops) into host cells (Derewenda et al., 2004, Fields et al., 1999, Pettersson et al., 1999), to having immunomodulatory properties of its own, such as inhibiting the production TNF-α and IFN-γ inflammatory cytokines (Leary et al., 1995).

In this study, the effect that *Yersinia pestis* V antigen and V antigen fractions have on the innate immune system was investigated, with emphasis on PRRs, signalling pathways and ultimately cytokine secretion.

The innate immune system is the host's quick response mechanism against invasive pathogens. It does so through many mechanisms but the most intricate function of the innate immune system involves pattern recognition receptors (PRRs), which are able to recognise certain pathogen-associated molecular patterns (PAMPs) that are present on all pathogens. Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are part of the PRR family and are responsible for the detection of a range of PAMPs, such as cell wall components like lipopolysaccharide (LPS) from Gram-negative bacteria to viral DNA or single stranded RNA. PAMP recognition through PRRs causes the activation of intracellular signalling pathways that ultimately cause the release of inflammatory mediators such as TNF, IL-1β and IL-6 (Kawai and Akira, 2011, Kumar et al., 2011). The field of TLR research is relatively new with the first human Toll being discovered in 1997 by Medzhitov and Janeway. Our knowledge of the role that PRRs have in pathogen recognition and disease has been expanding as throughout the years, making them exciting targets for new therapeutics.

PRR activation typically triggers a signalling cascade that results in differences in gene expression and secretion of inflammatory mediators, such as cytokines, to promote the appropriate response against the detected pathogens. Some pathogens, however, such as *Yersinia pestis*, are capable of subverting these mechanisms for their own survival and proliferation. This study was interested in the ability that *Y. pestis* V antigen and V antigen fractions have in modulating the innate immune system.

In order to determine the effects of V antigen and V antigen fractions on the innate immune responses, initially the recombinant proteins were generated. The recombinant LcrV plasmids (pVG110) with a glutathione S-transferase (GST) were kindly provided by Professor Richard Titball (Chemical and Biological Defence Establishment, Porton Down, Salisbury, UK) and transformed into *E. coli* BL21 cells as previously described by (Carr et al., 1999). In order to produce V antigen fractions (V1, V3, V4 and V5), the recombinant protein plasmid (pGEX6P-1), again with a glutathione S-transferase (GST) tag, were kindly provided by Dr. Claire Vernazza (Defence Science and Technology Laboratory, Porton Down, Salisbury, UK). The plasmids were transformed into *E. coli* BL21 cells as previously described by (Carr et al., 1999).

After purification using affinity chromatography, both V antigen and V antigen fractions were passed through a Profos Endotrap® blue 10 (Hycult Biotechnology) to ensure there was no LPS in the final solution. This was verified using the *Limulus* Amebocyte Lysate (LAL) assay), which did not detect any traces of LPS in the purified preparations.

In the present study we investigated the effects of V antigen have on PRR expression, signalling pathways and cytokine release in THP-1 and RAW 264.7 cells.

4.1. Effects of V antigen in innate immune responses

Having shown that recombinant V antigen was free of contaminants, its effect in innate immune responses was investigated. This study utilised human monocytic leukemia (THP-1) and mouse leukaemic monocyte macrophage (RAW 264.7) cells to elucidate PRR expression, signalling pathways and inflammatory cytokine release in response to LPS, and if V antigen was able to modulate the response. Initially we investigated PRR expression in response to LPS via indirect immunofluorescence and flow cytometry. It was shown that LPS caused up-regulation of TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12 expression on THP-1 cells. Increased TLR1 and NLRP1 expression was not observed in any stimulation. Augmented TLR, CD14, NOD and NLRP expression has previously been observed in response to LPS (Akira et al., 2006, An et al., 2002, Kawai and Akira, 2009, Mao et al., 2013, Raetz and Whitfield, 2002, Takahashi et al., 2006). Results from RAW 264.7 cell line show that LPS caused up-regulation in expression of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14, NOD1, NOD2, NLRP3 and NLRP12.

When investigating what effect V antigen had on PRR expression in THP-1 and RAW 264.7 cells it was found that V antigen was able to modulate the expression of PRRs in both cell types. In particular, V antigen seemed to induce a general downregulation of all PRRs tested in RAW 264.7 cells. In the case of THP-1 cells V antigen seemed to downregulate most PRRs with the exception of TLR9, CD14, NOD2, NLRP1 and NLRP3, where an up-regulation was observed.

The current findings are in contrast to the studies of Sharma *et al.* (Sharma et al., 2005, Sharma et al., 2004) and Reithmeier-Rost et al. (2004) where they observed no difference in TLR4 and an up-regulation of TLR2 and TLR6 in the former and no difference in TLR2 and TLR4 expression following V antigen stimulation in the later study. Differences observed between the different studies could be due to the fact that

the Sharma *et al*. study was performed in Balb/c mice peritoneal macrophages whereas this study was performed in human monocytic leukemia (THP-1) and mouse leukaemic monocyte macrophage (RAW 264.7) and they were observing effects after 12 hour stimulation. In the case of Reithmeier-Rost *et al*. study, the strain of Yersinia was completely different, as they used *Y. enterocolitica* O8 rLcrV, whereas in this study *Y. pestis* LcrV was used. It has been previously shown that LcrV from *Y. enterocolitica* and LcrV from *Y. pestis* can have different effects from each other (Abramov et al., 2007, Reithmeier-Rost et al., 2004, Sing et al., 2002a).

Having shown that stimulating cells solely with V antigen modulates PRR expression in both cell types, the next step was to investigate whether pre- or post-incubating with V antigen can alter the innate immune response of THP-1 and RAW 264.7 cells in response to LPS.

It was found that pre-incubating THP-1 cells with V antigen followed by LPS stimulation resulted in a up-regulation of TLR2, TLR6, TLR7, CD14, NOD1, NOD2, NLRP1, NLRP3, NLRP12 expression. There was no change in TLR4 and a small downregulation in TLR9 expression. On the contrary, when RAW 264.7 cells were pre-incubated with V antigen TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, CD14 and NOD1 expression decreased. There was no change in NLRP1 and NOD2 expression where as a slight increase in NLRP3 and NLRP12 was seen.

When THP-1 cells were post-incubated with V antigen there was a reduction on TLR2, TLR4, TLR6 and TLR7 expression. On the other hand, TLR9, CD14, NOD1, NOD2, NLRP1, NLRP3 and NLRP12 expression levels seem to increase. Our results show a different outcome in PRR expression levels when RAW 264.7 cells are post-incubated with V antigen - every PRR investigated in this study seem to reduce its expression in response to V antigen. This disparity between the two cell types might be because monocytes (THP-1 cells) are released into the circulation where they migrate into most

tissues, due to growth factors, inflammatory cytokines and microbial products, they differentiate into mature macrophages (RAW 264.7 cells). The main function of monocytes is to replenish the levels of circulating macrophages, whereas mature macrophages have a more active role in immune activities such as phagocytosis and immune suppression (Murray and Wynn, 2011, Shi and Pamer, 2011). Perhaps *Yersinia pestis* V antigen has evolved to immunosuppress certain cell types such as macrophages rather than monocytes, which may pose a more direct threat to *Y. pestis* survival and proliferation.

Having shown the effect of V antigen on PRR expression, the next step was to determine how this protein influenced NF-κB and caspase-1 activation in response to LPS. This was assayed by either pre- or post-incubating with V antigen and measuring the intracellular concentration of phosphorylated IκB and caspase p-10. Our results in THP-1 cells show that V antigen inhibits phosphorylation of IκB, therefore NF-κB. Additionally our results show a reduction in caspase-1 p-10, thus caspase-1 activity. In RAW 264.7 cells, our results demonstrated that V antigen reduces both phosphorylation of IκB and caspase-1 p-10, therefore NF-κB and caspase-1 activity, respectively. These findings are in agreement with the study of Sodhi et al. (2004), where they have also observed NF-κB inactivation in response to V antigen.

Given that V antigen was found to modulate the expression of PRRs and the activation of both NF-κB and caspase-1 activation, the next step in this study was to examine the release of cytokines, the inflammatory mediators, caused by LPS and whether or not V antigen was able to modulate their secretion. Inflammatory cytokines were measured in the cell supernatant using flow cytometry coupled with cytokine bead array system. Cytokine analysis of THP-1 and RAW cells in response to LPS gave notable increase in TNF, IL-6, IL-1β and IL-10 in comparison to unstimulated cells. When it was investigated whether V antigen was able to trigger any cytokine secretion on its own, our results showed no marked difference in cytokine secretion levels.

Pre- or post-incubating V antigen in response to LPS was also tested. It was shown that in both THP-1 and RAW 264.7 cells pre-incubation with V had little or no effect on the release of cytokines.

When THP-1 cells were post-incubated with V antigen TNF and IL-1β levels seemed to be completely inhibited. IL-6 secretion seems to be inhibited in the first 3 post-incubation time-points and an acute increase occurred at the 6 post-incubation hour. This might be due to the fact that V antigen is no longer effective after 6 hours of LPS stimulation, or more specifically that it is not able to combat the huge amount of IL-6 that was accumulated 6 hours after LPS challenge. IL-10 cytokine production seemed to be up-regulated in response to V antigen. It increased progressively through the time-points reaching a maximum at the 6 hour post-incubation mark. This finding is in agreement with the study of Reithmeier-Rost et al. (2004) and (Sharma et al., 2005), suggesting that V antigen is able to trigger anti-inflammatory cytokine production and thus immunosuppress the innate immune response.

Our RAW 264.7 cells show that pre- or post-incubating with V antigen resulted in a downregulation of TNF, IL-6, IL-1β and IL-10 in comparison with cells stimulated with LPS alone.

Overall our data suggests that V antigen is able to modulate PRR expression as well as inhibit signalling cascades and pro-inflammatory cytokine secretion in RAW 264.7 cells. When compared to THP-1 cells the results are similar, but rather more pronounced inhibition is seen in RAW 264.7 cells.

4.2. Effects of V antigen fragments in innate immune responses

Previous studies have shown that, not only the entire, but also small regions of *Yersinia pestis* V antigen can provide protection against the plague. It has been suggested that *Y. pestis* V antigen contains a number of protective epitopes that are located in a central region between amino acids (a.a.) 135 and 275 (Hill *et al.*, 1997). In this study, out of the six fragments that possibly could confer protection, proposed by Vernazza et al. (2009), four were chosen – V1, V3, V4 and V5. V1 was selected because it had been previously shown to be protective against plague, V3 due to a N-terminal deletion whereas V4 and V5 a C-terminal deletion type. In order to determine the efficacy of the different V antigen fractions, NF-κB and caspase-1 activation and the cytokine secretion was investigated.

Initially, we tested if RAW 264.7 cells would activate NF-κB and caspase-1 when stimulated with LPS. Our results showed that both cell types do activate both NF-κB and caspase-1.

Once it was established that LPS was able to trigger the NF-κB signalling cascade and caspase-1 p-10, whether the different V antigen fractions were able to modulate this response was investigated. Our results revealed in RAW 264.7 cells V1, V4 and V5 inhibited NF-κB and caspase-1 activation over time. V3 fraction was able to decrease NF-κB and caspase-1 activation, but not to the same extent as the other fractions.

The next step was to evaluate the inflammatory cytokine release and if the different fractions had an effect in the levels secreted by RAW 264.7 cells. Cytokine analysis of RAW 264.7 cells in response to LPS gave notable increase in TNF, IL-6, IL-1β and IL-10 in comparison to unstimulated cells. Our results show V1, V3, V4 and V5 can inhibit the production of TNF, IL-6, IL-1β and IL-10 inflammatory cytokines. The highest reduction of cytokine release was seen with V1 followed by V4, V5 and last V3,

therefore the functional domain of V antigen responsible for the effects that we have observed lies within the amino acids 135-275.

Out of the fragments analysed, V3 seemed to have the least immunosuppressive properties. This might suggest that residues within a.a. 135-175 are important for the stability of the epitope, which results in a lower immunomodulation capability.

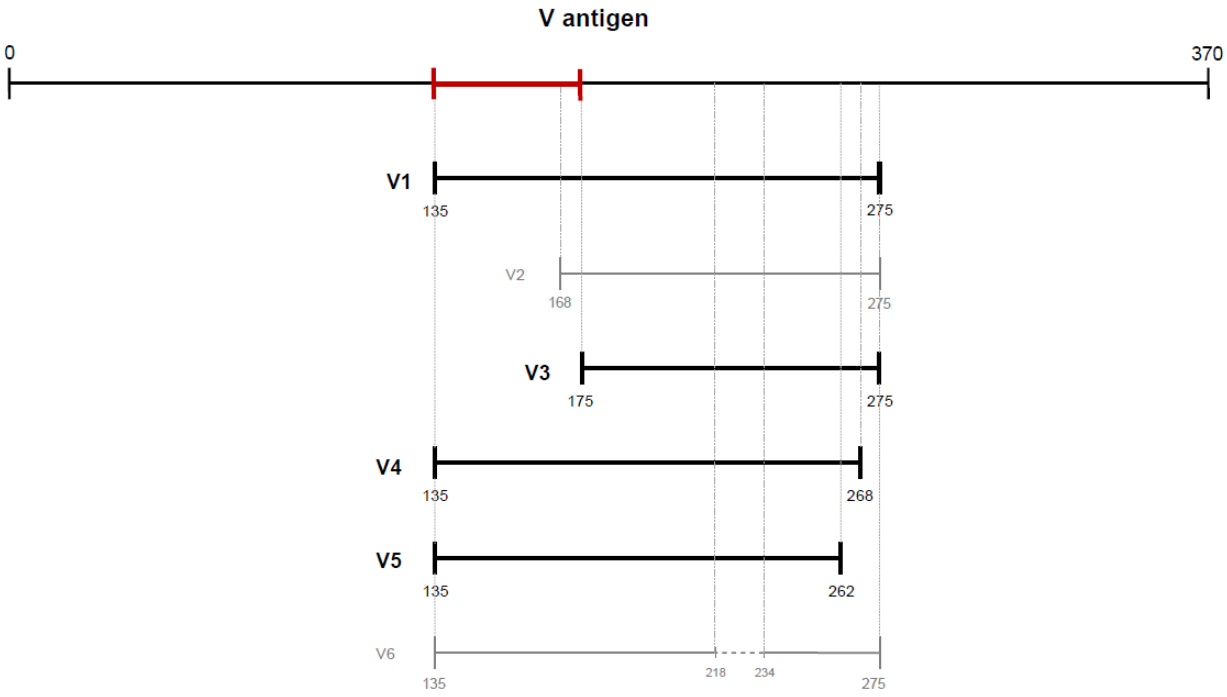


Figure 4.1: Schematic representation of V antigen and its fragments V1, V2, V3, V4, V5 and V6. In this study, the fragments selected were V1 (a.a. 135-275), V3 (a.a. 175-275), V4 (a.a. 135-268) and V5 (135-262). a.a. 135-175 (in red) might be important for the stability of the epitope.

4.3. Intracellular trafficking and targeting of exogenous V antigen

The innate immune system has an essential role in recognising molecular “signatures” of invading pathogens. It does so through a range of receptors which are tailored to recognise different molecular ligands. This study was concerned with the effects of V antigen has in the innate immune system. It is becoming apparent that the presence of V antigen may have the effect of altering cellular response to LPS.

Our data has shown that V antigen interferes with PRRs, ultimately leading to a modified cellular response. Thus we proceeded to employ triple label confocal microscopy in order to image whether V antigen from *Y. pestis* associates with PRRs, signalling molecules and cellular organelles. In order to track V antigen within the cell, it was conjugated to a fluorescent tag – Oregon Green (OG) or FITC.

Confocal microscopy was used in order to test the effect of V antigen on TLR4 and MyD88 in response to LPS.

It was shown that unstimulated cells and cells incubated with V antigen by itself did not activate TLR4 or MyD88. On the other hand, when RAW 264.7 cells were stimulated with LPS, both TLR4 and MyD88 showed, as expected, activation and co-localisation. When cells were stimulated with LPS and post-incubated with V antigen our results show that in the first two post-incubation periods there is still activation and co-localisation of both TLR4 and MyD88, but by the 4 and 6 post-incubation time-point, TLR4 and MyD88 co-localisation seems to be inhibited. Interestingly, at the 2 hour post-incubation mark, MyD88 seems to cluster with each other. These results might suggested that V antigen is interfering with the normal function of MyD88, which would result in an interference with subsequent downstream signalling.

Additionally, confocal microscopy was also used in order to test V antigen colocalisation with TLR4, NLRP3 and NLRP12. Our results show that that V antigen is co-localising with NLRP3 and NLRP12 immediately within 10 minutes of stimulation

and throughout the 2 hours. These results seem to be in agreement with Vladimer et al. (2012) where both NLRP3 and NLRP12 were linked to the recognition of *Yersinia pestis*. This co-localisation might result in the prevention of the assembly of the inflammasome complex, resulting in the inhibition of IL-1β seen in this study.

The co-localisation of MyD88, IRF3 and IRF7 with V antigen were also investigated.. Our results show that both MyD88 and IRFs co-localised with V antigen, demonstrating that V antigen not only interacts with the dependent (MyD88) but also independent (IRF3 and IRF7) pathways. This might be linked to the fact that V antigen might be meddling with both dependent and independent pathways, resulting in the inhibition of inflammatory cytokines as seen above.

Finally, cells were incubated with V antigen and labelled for EEA1, a protein within the early endosomes. Our results show that V antigen associates with EEA1, which shows that it might be concentrated within endosomes. This might be linked to the internalization route that V antigen follows when it is internalized within the cell.

4.4. *Yersinia pestis* V antigen as a therapeutic intervention for sepsis

Throughout this study it has been shown that V antigen is able to modulate the innate immune responses in response to LPS. In particular, V antigen has shown to reduce dramatically the main pro-inflammatory cytokines – TNF and IL-1β. It is widely accepted that the onset of sepsis results from an over-reaction of the host that occurs at the level of the innate immune system and is directly linked to the recognition of bacterial cell wall components, such as lipopolysaccharide (LPS) from Gram-negative bacteria. Thus the reduction in this excessive production of pro-inflammatory cytokines might be the key to a possible therapeutic intervention.

It is hypothesised that the modulation of this immune response could have a therapeutic potential. We went on to explore the blocking of the endogenous and microbial sensing apparatus *in vivo* to determine the beneficial effects of V antigen as a

prophylactic and also therapeutic intervention in the reversing of the detrimental outcomes septic patients portray. A male CD-1 mouse model of sepsis was used to test whether or not V antigen is able to prevent the over-expression of pro-inflammatory cytokines, as a result of sepsis, and ultimately survival.

Intraperitoneal (i.p.) injection of V antigen was found to be very effective at decreasing mortality in CD-1 male mice. Mice pre-treated with V antigen improved survival by 70% over the 72 hours. V antigen was most effective at preventing septic shock when mice were post-treated with the protein, where 80% survival was observed at 72 hours. Interestingly it was shown that the longer the treatment was postponed the better, as the 4 and 6 h post-treatment gave us the best survival rates, this might be due to the fact that V antigen has more time to have its full immunomodulatory effect. The blood concentrations of TNF and IL-1β were all reduced after mice were pre- or post-treated with V antigen in comparison to those that were not. A possible mechanism for the modulatory effect of V antigen on inflammatory response both *in vitro* and *in vivo* might be on its ability to disrupt the MyD88 adaptor molecule disruption, resulting on a direct interference of the downstream signalling.

Adult CD1 outbred mice have been extensively in order to study the development of early septic-shock syndrome that causes high mortality (Doi et al., 2009, Dominguez-Punaro Mde et al., 2008, Rodriguez et al., 2009, Wang et al., 2003, Zager et al., 2003). Several different interventions have been tested on the CD1 sepsis model in order to assess whether they can improve survival or tissue injury, but none of the interventions have demonstrated the extent of improved survival as well as the inhibition of cytokines that we have shown (in the same model). Interventions that have been tried include treatment with anti-hypercholestromic agents, such as Cerivastatin (Ando et al., 2000), blockade of receptors such as A2B adenosine receptor blockade (Belikoff et al., 2011), CXCR2 (Ness et al., 2003) or high mobility group box protein-1 (HMGB1) (Leelahavanichkul et al., 2011) as well as using Androstenediol (androst-5-ene-

3β,17βdiol; 5-AED), a natural adrenal steroid (Nicoletti et al., 2010) – none of these interventions showed the promise that V antigen has shown with almost 80% improvement in survival and almost complete inhibition of pro-inflammatory cytokines.

These findings are very exciting, since they demonstrate that V antigen could be used effectively as a prophylactic intervention against sepsis and other inflammatory conditions, but most importantly it can be used in the future as a therapeutic intervention for the cytokine storm observed in patients with sepsis.

4.5. Concluding remarks

Sepsis is a medical condition that stems from a multi-system disorder resulting in a loss of homeostasis which in turn results in multi-organ failure and a high chance of death. It is an area of unmet clinical need and thus there are very few new products that are reaching the market. It is imperative to seek out new therapeutic interventions in order to modify this severe outcome.

V antigen can clearly modulate the innate immune response. Throughout this study we have shown the capability of this protein to modulate PRR expression, signalling pathways, cytokine secretion and *in vivo* survival.

Our data show for the first time that V antigen can inhibit the production of TNF and IL-1β which are crucial on the onset of sepsis. Of a greater magnitude, our *in vivo* data show an impressive 80% reduction in mortality when mice are treated with V antigen. This protein should unquestionably be further investigated as a possible therapeutic intervention for sepsis and future studies should focus on testing not only the whole V antigen protein, but peptides encoding the functional domain of V as a therapeutic intervention not only for sepsis but for other inflammatory conditions as well.

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