The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

School of Veterinary Medicine and Science

The Therapeutic Potential of Vasoactive Intestinal Peptide (VIP) in the Treatment of Gram-Negative Sepsis

By

Basim Ali Askar

BVSc, MVSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

School of Veterinary Medicine and Science

April 2016

Abstract

Gram-negative bacteria are the most common cause of the sepsis and lipopolysaccharide (LPS) a major component of Gram-negative bacteria is known to be of major importance in the development of sepsis. Human infection with *Salmonella*, a Gram-negative bacterium, is associated with a number of cases of sepsis and is particularly important in childhood sepsis.

During salmonellosis, monocytes and macrophages produce a number of different pro-inflammatory mediators such as TNF-α, IL-1α, IL-12, IL-18, IFN-γ, reactive nitrogen species and oxygen species. Although the production of these inflammatory mediators is required for resolution of bacterial infections, they are contraindicated in diseases such as sepsis. In the initial (acute) phase of sepsis a Systemic Inflammatory Response Syndrome (SIRS) occurs in which inflammatory mediators are produced in high concentration, which can lead to organ failure and death. The SIRS phase is then replaced by a Compensatory Anti-inflammatory Response Syndrome (CARS) phase which leads to immunosuppression. The CARS phase can lead to secondary infection and subsequent mortality within 28 days of hospital admission.

To date, several studies have evaluated the role of vasoactive intestinal peptide (VIP) as an anti-inflammatory agent that may have therapeutic potential in septic patients both *in vitro* and *in vivo*. VIP has been shown to inhibit production of inflammatory mediators

produced by human monocytes in response to LPS. The aim of the work described in this thesis was to investigate the therapeutic potential of VIP in sepsis using an *ex vivo* human monocytes model infected with viable *Salmonella Typhimurium* 4/74 (rather than LPS).

The study shows that VIP (10^{-7} M) stimulates an increase in the numbers of *Salmonella* recovered from infected human monocytes (MOI = 10). In addition, VIP also increases the survival rate of human monocytes infected with *Salmonella*. These two results may suggest a detrimental effect of VIP during bacteraemia and sepsis, since monocyte death may be beneficial during sepsis and bacterial overgrowth could lead to further increased LPS (and other antigen) stimulation of the immune system. However, VIP did significantly decrease *Salmonella* and LPS-induced TNF-α, IL-1β and IL-6 in monocyte supernatants. VIP also had a positive effect on IL-10 production in human monocytes infected with *Salmonella* or stimulated with LPS. Whether this suggests a possible detrimental effect of VIP is unknown but septic patients with high serum IL-10/ low TNF-α concentration ratio have previously been shown to have a poor prognosis. Higher IL-10 concentrations in infected monocytes (due to VIP) could also increase the CARS phase of disease with increased immunosuppression.

Flow cytometry and qPCR analyses showed that of all of the VIP receptors, VPAC1 was expressed most highly during *Salmonell*a infection, or LPS stimulation, of human monocytes. Administration of

VIP inhibited VPAC1 has been shown by many studies to be the most important receptor by which VIP inhibits production of inflammatory immune mediators, or increases IL-10 production from murine macrophages. Results in this thesis, therefore, suggested that *Salmonella* infection may promote VPAC1 expression and so provide a mechanism of inhibiting the production of inflammatory mediators in infected cells. This could then increase intracellular survival of *Salmonella* and provide a means of greater dissemination of the infection.

To ascertain how increased VPAC1 expression on the surface of monocytes may be achieved, analysis of the expression of known intracellular endosomal and exosomal constituents was performed. Confocal laser microscopy, using specific antibodies, showed that VPAC1 on the monocyte cell membrane was internalised within early endosomes (measured by co-localisation of VPAC1 and EEA1) rather than being degraded within lysosomes (measured by immunoreactivity to LAMP1). VPAC1 is then transported via a Rab11A recycling endosome and packaged in the Trans-Golgi network (TGN), shown by co-localisation of VPAC1/Rab11A and the TGN marker (TGN46). VPAC1 was then associated with Rab3a and calmodulin. The function of these latter two proteins in the docking of exosomes to the cell membrane is well known, thus suggesting that *Salmonella* induced VPAC1 was also recycled to the cell membrane within exosomes. VIP inhibited the expression of both Rab3a and calmodulin but not the co-localisation of VPAC1 with these two proteins. Further studies then

showed that a calmodulin agonist (CALP1) increased VPAC1 expression on the surface of monocytes, while a calmodulin antagonist (W-7) decreased expression of VPAC1 on the surface of monocytes.

In conclusion, this thesis does present hitherto unknown data regarding *Salmonella* infection of human monocytes and the effects of VIP on infected monocytes. VIP has potential as an anti-sepsis therapy since it reduces the production of inflammatory mediators by *Salmonella*-infected and LPS-stimulated monocytes. However, the fact that VIP increases survival of infected human monocyte and increased growth of *Salmonella* in human monocytes may preclude its use in sepsis.

Declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Nottingham.

The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

Name: Basim Ali Askar

Signature: 

Date: 21 April 2016

Conferences and Meetings

1. **Immunology Showcase**, 20th June 2013, Medical School University of Nottingham, UK (attendance)
2. **MHS Faculty Postgraduate Research Forum**, 25th of June 2014, the University of Nottingham, UK (attendance).
3. **M5 Biomedical Imaging Conference**, 9th September 2014, the University of Nottingham, UK (poster presentation).
4. **Kurdistan Students Conference**, 15th September 2014, the University of Nottingham, UK (attendance).
5. **The Innate Immunity Summit,** 10-12 November 2014, the O2 Peninsula Square, London, UK (poster presentation).
6. **The international Sepsis Forum**, 2nd-5th December 2014, Pasteur Institute, Paris, France (poster presentation).
7. **The Flow Cytometry Meeting**, 12th of November 2014, the University of Nottingham, UK (attendance).
8. **Immunology Showcase**, 22th January 2015, Medical School, the University of Nottingham, UK (attendance).
9. **The 2015 Immunotherapy**, 12 March 2015, Watershed, Bristol, UK (poster presentation).
10. **Tissue Inflammation and Local Immune Responses**, 17th of April 2015, the John Radcliffe Hospital, Oxford, UK (poster presentation).
11. **Midlands Sequencing Consortium Meeting** 13th July 2015, the University of Nottingham, UK (attendance).
12. **9th European Workshop on Immune-Mediated Inflammatory Diseases**, 2nd – 4th September 2015, Amsterdam, the Netherlands (oral presentation).

Publications

Askar B, Ibrahim H, Barrow B and Foster N (2015). Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune responses in human monocytes infected with viable *Salmonella* or stimulated with LPS. *Peptides*, 71**,** 188-195.

Manuscripts submitted

Ibrahim H, Askar B, Barrow B and Foster N. Dysregulation of JAK/STAT genes by VIP in *Salmonella*-infected monocytes may inhibit its therapeutic potential in human sepsis. Journal of Elsevier /peptides (submitted Manuscript 2015).

Acknowledgements

First and foremost I would like to take this opportunity to thank God, The Almighty, for being my strength and guidance to complete this task. Without Him, I could not have had done this.

I want to express my especial appreciation to Dr Neil Foster for his excellent support and care throughout this work. I would like to thank you for encouraging my research and for allowing me to grow as research scientist. Your advice on both research as well as my career has been invaluable. Similar, profound gratitude goes to Prof. Paul Barrow for the amazing experience and being a major source of advice. It was a great privilege and honor to work and study under your guidance.

I am also very grateful to Scott Hulme, Belinda Wang and Paula William for excellent lab training assistance.

I would like to extend thanks to Hiba Ibrahim, Luke Richard, John Higgins and all colleagues who helped me with lab technique skills.

I am also indebted to all my family members in Iraq. My parents who have been a constant source of motivation to pursue my goals. My brothers Kheder, Dilshad, Naeim, Nasim and Wissam for the support and encourage during my time here.

To my lovely wife “Suzan” as well as my beloved daughter “ Elina” and also my new-born son“ Ian”. They were always there supporting me and stood by me through the good and bad times. I am hugely appreciative to my uncle, Hassan, for providing support and whatever I needed.

To my lifelong friends Azad and Shaker who always cheered me up and whoever have helped me.

I would like to express my deepest gratitude to the Ministry of Higher Education and Scientific Research, Kurdistan Region of Iraq, particularly Professor Dlawer Ala 'Aldeen, for funding study and living expenses during my PhD period. Finally, especial thanks to the University of Nottingham for hosting this project.

Basim

Contents

List of Tables

List of Figures

List of Abbreviations

S. Typhimurium	Salmonella enterica serovar Typhimurium
VIP	Vasoactive intestinal peptide
SPI	Salmonella pathogenicity islands
TTSS	Type three secretion system
SCV	Salmonella containing vacuole
MOI	Multiplicity of infection
MHC	Major Histocompatibility Complex
LPS	Lipopolysaccharide
LPB	LPS binding protein
TLR	Toll like receptor
NFκB	Nuclear factor kappa B
GPCR	G-protein coupled receptors
PHI	Peptide histidine isoleucine
PHM	Peptide histidine methionine
PACAP	Pituitary adenylate cyclase activating polypeptide
PAC1	PACAP Receptor1
VPAC1	Vasoactive intestinal polypeptide receptor 1
VPAC2	Vasoactive intestinal polypeptide receptor 2
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
PKA	Protein kinase A
JNK	Jun N-terminal kinases
AP.1	Activator protein 1
MEKK1	Mitogen-activated protein kinase kinase kinase
TNBS	Trinitrobenzene sulfonic acid
CFU	Colony-forming unit.

IL-1β	Interleukin-1 beta
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
NO	Nitric oxide
ROS	Reactive oxygen species
iNOS	inducible nitric oxide synthesis
NADPH	Nicotinamide adenine dinucleotide phosphate
SIRS	Systemic inflammatory response syndrome
CARS	Compensatory anti-inflammatory response syndrome
M cells	Microfold cells
TTSS	Type three secretion system
APCs	Antigen-presenting cells
DCs	Dendritic cells
NK	Natural killer
Treg T cells	Regulatory T cells
TH1	Type 1 helper T cells
PEEC	Pathogen-elicited epithelial chemoattractant
PP	Peyer's patches
MLN	Mesenteric lymph nodes
PMNs	Polymorphonuclear leukocytes
MIG	Monokine induced by gamma interferon
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PRRs	Pathogen recognition receptors
PAMPS	Pathogen-associated pattern recognition receptors
LBP	LPS binding protein
NOD	Nucleotide-binding oligomerization domain
NLRs	Neucleotide binding domain/leucine rich repeat

THP-1	Human monocytic
TIR	Toll-interleukin 1 receptor
TIRAP	Toll-interleukin 1 receptor (TIR) adaptor protein
MyD 88	Myeloid differentiation factor 88
IRAK1	Interleukin-1 receptor-associated kinase 1
TRIF	TIR domain-containing adaptor-inducing IFN-beta
TRAM	TRIF-related adaptor molecule iκB kinase (IKK)
MAPK	Mitogen activated protein kinase
AP-1	Activator protein-1
TRAF6	TNF-α receptor- associated factor 6
ERKs	Extracellular signal-regulated kinases
JNK	c-Jun N-terminal kinase
PKC	Protein kinase C
TGF-β	Transforming growth factor-β
STAT	Signal transducer and activator of transcription
Ca^{2+}	Calcium
CaM	Calmodulin
CaMK	CaM kinase
PLC	Phospholipase C
PAF	Platelet-activating factor
HMGB-1	High-mobility group box-1
InsP3	Inositol trisphosphate
PKA	Protein kinase
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding
GPCR	G protein-coupled receptors
GRK	GPCR kinase
AC	Adenylyl cyclase

Rab3a	Ras-associated protein 3a
SNARE	Soluble N-ethylmaleimide proteins receptors
RIM	Rab3-interacting molecules
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GDI	GDP dissociation inhibitor
MDMs	Human monocyte-derived macrophages
MDDCs	Human monocyte-derived dendritic cells
BMDDCs	Murine bone marrow derived dendritic cells
Caspases	Cysteine-aspartic proteases
KO	Knockout
NLR	Nucleotide binding domain/leucine rich repeat
JAK1	Janus tyrosine kinases
ICAM-1	Intercellular adhesion molecule-1
VCAM -1	Vascular cell adhesion molecule-1
COS7	African monkey kidney
HEK	Human embryonic kidney
CHO	Chinese hamster ovary
EEA1	Endosomal antigen1
LAMP1	Lysosomal associated-membrane proteins1
TGN	trans-Golgi network
RA	Rheumatoid arthritis
PBS	Phosphate buffer saline
MACS	Magnetic-activated cell sorting
FACS	Fluorescence Activated Cell Sorting
PI	Propidium iodide
FSC	Forward scatter
SSC	Side scatter

ELISA	Enzyme-linked immunosorbent assay
DAPI	4',6-diamidino-2-phenylindole
FITC	Fluorescein isothiocyanate
BSA	Bovine serum albumin
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
gDNA	Genomic DNA
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris-acetate-EDTA
cDNA	Complementary DNA
qPCR	Quantitative PCR
ANOVA	Analysis of variance

Chapter One

1. General Introduction

1.1 Sepsis

Sepsis is a life threatening condition which etiologically occurs due to the inflammatory immune response to micro-organisms (Matot and Sprung, 2001). Acute sepsis is characterised by severe physiological changes in the septic host. These changes include either increase or decrease in body temperature, heart rate and volume of leukocytes (Jacobi, 2002). Severe sepsis occurs following acute sepsis and is associated with multiple organ dysfunctions such as renal, hepatic or cardiac failures, oliguria, coagulation abnormalities, hypoxia and lactic acidosis (Nguyen et al., 2006). Severe sepsis is usually fatal occurs 1-2 weeks from the onset (reviewed in Ulloa and Tracey, 2005). Moreover, septic shock is applied when the case develops into unresponsive hypotension (Levy et al., 2003) and usually results in mortality 1 – 2 days from sepsis onset (Ulloa and Tracey, 2005). Severe sepsis remains a major cause of nosocomial mortality. It has a global impact on about 18 million individuals (Ulloa and Tracey, 2005) and causes mortality rates as high as 30 % to 50 % in intensive care units (Dellinger et al., 2008).

Existence of bacteria within the blood circulation called bacteraemia whereas the bacterial dissemination within the body associated with systemic immune reaction against microorganism is known as septicaemia (Chaudhary et al., 2013). Septicaemia is an important

cause of morbidity and mortality in African children with death rate between 30 – 70% depending on many factors such as virulence of the microorganism and host immune system (Wenzel et al., 1996). The amount of bacteraemia, by culturing blood, is higher in children than adults and death rate is positively correlated to the magnitude of bacteraemia. Mortality due to Gram-negative bacteraemia was 50% in patients' blood with less than 5 CFU/ml while the death rate increased to around 85% when bacterial growth contained up to 5 CFU/ml (Yagupsky and Nolte, 1990).

Blood culture is an important part in the management of sepsis for rapid quantification and identification of the causative agents. To increase identification of the pathogen, it is recommended using aerobic and anaerobic bottles (Dellinger et al., 2008). Culturing of the blood in septic patients obtain approximately 30-50% positive (Raad et al., 2007). Burden and type of pathogen and also the requirements for bacterial growth are essential to determine the positivity of a blood culture (Peralta et al., 2006). Several blood culturing methods have been used for enumerate bacterial growth. Broth culture media largely depends on the amount of blood withdrawn from patients. For large blood volume (>10 ml), pour plate and spread plate methods are recommended while for small volume (<1.5 ml) by direct plating through using heparin tubes and Isolator Microbial Tube. The latter method contains anticoagulant, sodium poly anetholsulfonate and saponin. The method causes lysis of the blood and then required centrifugation and plate on agar media (Reviewed in Yagupsky and

Nolte, 1990). Several commercial biochemical test have been greatly used for identification of the positive blood culture such as Vitek II, MALDI-TOF MS and Sepsityper kit (Morgenthaler and Kostrzewa, 2015).

In England and Wales, cases of sepsis increased from 18000 to 31000 from 1996-2006 and resulted in 11500 deaths (Harrison et al., 2006). In 1999 – 2005, it was the tenth greatest cause of mortality in the USA, being responsible for about 6% of the total recorded mortalities (Melamed and Sorvillo, 2009). Sepsis affects over 750000 people annually in the USA with a cost of 16.7 billion dollars per year and, it is estimated, that overall a global increase in sepsis occurs at a rate of about 1.5% annually (Angus et al., 2001). For this reason, sepsis is an important global problem, which not only causes significant morbidity and mortality but also places a substantial financial strain on health care provision. Infection by Gram-negative bacteria is the most common cause of the sepsis (reviewed in Martin, 2012) and lipopolysaccharide (LPS), also called endotoxin, is the main bacterial component responsible for the condition. LPS is the main component of the outer layer of Gram-negative bacteria and is in itself composed of three distinct parts, lipid A, the core and O antigen (Wong and Luk, 2009). Of these, lipid A, the innermost and highly conserved region, is the principal toxic element of the LPS (Kabanov and Prokhorenko, 2010). Lipid A is composed of two glucosamine bases connected to phosophate groups and hydroxyacyl chain fatty acids. The structures

differ according to the number and length of hydroxyacyl chains, which is responsible for its charge (Raetz et al., 2009).

Although LPS in the Gram-negative bacteria is the main factor responsible for induction of sepsis, peptidoglycan and lipoteichoic acid in Gram-positive bacteria can also synergise to induce sepsis (Sriskandan and Cohen, 1999). Both peptidoglycan and lipoteichoic have been suggested to trigger inflammatory reactions, cause multiple organ dysfunction and sepsis in many experimental models of *in vivo* and *in vitro* (Wang et al., 2003). Unlike in Gram-negative bacteria where recognise LPS by TLR4, TLR2 is the most crucial host recognition receptor for the bacterial cell wall components (Echchannaoui et al., 2002). It has been shown that peptidoglycan and teichoic acid of *Staphylococcus aureus*, which is regarded the most Gram-positive bacteria associated with sepsis (Fluit et al., 2000), triggered production of IL-1β, IL-6 and TNF-α in human monocytes but were up to 100 time less effective than LPS of *E. Coli* (Mattsson et al., 1993, Kimbrell et al., 2008).

Prostaglandin is also an important mediator for sepsis initiation. The production of prostaglandin is generated from arachidonic acid by enzymes called cyclooxygenase (Ricciotti and FitzGerald, 2011). In experimental models, it has been shown that administration of LPS greatly increase production of prostaglandin and thromboxane (Fink, 2001). Prostaglandin E_2 is the most prevalent type of prostaglandin that mediate several biological activities which results in inflammation

signs (Ricciotti and FitzGerald, 2011). Prostaglandin E_2 modulate the functions of several immune cells including DCs, macrophages also T and B cells causing both pro- and anti-inflammatory response (Egan et al., 2004). Leukotrienes are another inflammation mediator also derived from arachidonic acid. Leukotrienes have essential role in the recruitment of macrophages, neutrophils and lymphocytes into the inflammatory sites (Pierce et al., 1999) where they mediate tissue injury and development of the sepsis. Also, studies which used leukotrienes inhibitors have reported that the inhibitor reduced leukocytes adhesion to the vascular epithelium (Lee et al., 2013).

Exposure of the innate immune system to LPS leads to activation of transcription factors such as nuclear factor kappa B (NF-κB) and eventually the down-stream up-regulation of pro-inflammatory mediators. As a result, innate immune cells such as macrophages mediate production of several inflammatory cytokines such as IL-1β, IL-6, IL-10, IL-12 and TNF-α (Björkbacka et al., 2004, Kurt et al., 2008) as well as nitric oxide (NO) (Sade et al., 1999) when stimulated by LPS. Neutrophils recruitment to the inflammatory sites is also important in sepsis. During sepsis, the mechanism of recruitment of neutrophils become impaired and results in suppression of chemotaxis of neutrophils toward inflamed site (Arraes et al., 2006). Failure of neutrophil migration is complex and is believed to be mediated by cytokine production through TLR4 pathway. These inflammatory mediators induce iNOS production which in turn stimulate NO formation and cause deactivation of neutrophil chemotaxis (Alves-

Filho et al., 2008). In addition, there is crosstalk between natural killer (NK) cells and induction of sepsis. NK cells play essential role to initiate immune response against pathogen by having cytotoxic activities and production of pro-inflammatory mediators particularly INF-Y, TNF-α and GM-CSF. These mediators regulate the functions of immune cells including macrophages (Chiche et al., 2011, Souza-Fonseca-Guimaraes et al., 2013) and might induce the deleterious effects of the NK cells by enhancing tissue damage and then progression of sepsis (Fusakio et al., 2011).

Many studies have investigated the role of inflammatory mediators in sepsis. For example, Giamarellos-Bourboulis et al. (2011) analysed the levels of TNF-α, IL-1β and IL-6 in the serum of 92 patients with different stages of sepsis, detected by qPCR and enzyme immunoassay. In this latter study, the concentration of TNF-α was (40 pg/ml) higher than IL-1β and IL-6 (20:20 pg/ml), respectively and it was reported that the level of TNF-α was most closely correlated with the onset of septic shock. In rat cardiac macrophages, TNF-α is released 30 minutes after exposure to LPS and was shown to enhance NO production within 180 minutes (Grandel et al., 2000). Despite its short half-life (less than 20 minutes), administration of TNF-α into rats induced harmful physio-pathological conditions similar to sepsis. The pathological effect of TNF-α in these rats included endothelial damage, haemostasis, hypoxia, interstitial oedema and death from reduced cardiac output (Tracey and Cerami, 1994). These injurious effects of overwhelming production of TNF-α could be prevented by suppression

of TNF-α (Matot and Sprung, 2001). Later, Duncan et al. (2010) investigated the role of TNF-α and IL-1β on calcium level in the rat cardiac cells, measured by calcium indicator, fura 2. The group pointed out that increased production of cytokines resulted in escape of Ca^{2+} from ventricular myocytes and subsequently reduction in contractility, shortly followed by arrhythmia. It has also been reported that over production of NO and inducible nitric oxide synthesis (iNOS), by activated macrophages during sepsis, are also significant contributors to the pathogenesis of sepsis, causing endothelial injury, decreased vascular responsiveness, lower blood pressure and decreased cardiac output (Kirkebøen and Strand, 1999).

Several attempts have been made to neutralise the effects of endotoxin in both human and animal models of sepsis. However, to date, none of these produced satisfactory results. Clinical trials have tested many agents including corticosteroids, antibodies against LPS / lipid A (Oh, 1998) as well as anti TNF-α, anti-thrombin, insulin and blood perfusion systems (Opal, 2007). Currently antibiotic therapy is the treatment of choice. However, bacteria may develop resistance against them. Resistance to cephalosporins and fluoroquinolones (Acheson and Hohmann, 2001), streptomycin and tetracycline (Musgrove et al., 2006) have been reported for *S*. Typhimurium and also administration of combinations of such antibiotics may lead to multi drug resistance. Furthermore, in the case of severe sepsis, mortality is due to dysregulation of the inflammatory immune response and death occurs even after antibiotics have cleared the bacterial

infection (Hotchkiss and Karl, 2003, Devi Ramnath et al., 2006). This phase of disease is known as systemic inflammatory response syndrome (SIRS) but is followed by a compensatory anti-inflammatory response syndrome (CARS) which can then provide a platform for nosocomial infection (Annane et al., 2005, Ward et al., 2008). Therefore, discovering an alternative novel therapeutic agent which targets the initial 'cytokine storm' could diminish mortality from severe sepsis but the timing of anti-inflammatory intervention may also be crucial to the prognosis of septic patients, since administration of anti-inflammatory agents during the CARS phase of disease could have a detrimental effect.

Some studies have suggested that a 6 folds increase in the concentration of endogenous vasoactive intestinal peptide (VIP) occurs in human (Brandtzaeg et al., 1989) and murine serum during sepsis (Zamir et al., 1992). Based on these findings, many studies have evaluated the role of VIP as a potential therapeutic agent in sepsis, in both *in vivo* and *in vitro* and the possible role of VIP as a therapeutic agent in sepsis has, therefore, been studied in this thesis.

1.2 *Salmonell*a as model of Gram-negative sepsis

Salmonella enterica are Gram-negative, facultative, motile, non-lactose fermenting and non-spore forming bacilli which belong to the Enterobacteriaceae family of bacteria (Salyers, 2002). *Salmonella enterica* infect a vast range of animals such as mammals, rodents, reptiles, primates, fishes and birds (Seepersadsingh and Adesiyun, 2003, Jang et al., 2008) and cause significant global diseases (Majowicz et al., 2010). *Salmonella enterica* have been divided into more than 2,500 serovars (Popoff, 2001) on the basis of biochemical characteristics and surface antigen compositions which include somatic (O) antigen and flagellar (H) antigen (Voogt et al., 2002).

Human salmonellosis mainly occurs following consumption of infected food (Foley and Lynne, 2008) such as ingestion of undercooked meat, eggs, unpasteurized dairy products and contaminated water (Tauxe et al., 1997). The clinical signs of the disease usually appear 6 – 48 hours post infection (Arda et al., 2001) and presents as a self-limiting gastroenteritis (non-typhoid strains) mainly caused by *S*. Typhimurium and *S*. Enteritidis (Altekruse et al., 1997). These clinical signs generally include nausea, vomiting, abdominal cramps, diarrhoea and fever (Salyers, 2002). Moreover, bacteraemia and septic shock may occur in about 5-8% of non- typhoidal *Salmonella* infections (CHERUBIN et al., 1974, Shimoni et al., 1999). *Salmonella* can be excreted in the stool of humans for up to 1 year (Buchwald and Blaser, 1984, Murase et al., 2000). Over 73000 cases of non-typhoidal salmonellosis were reported in the UK (England and Wales) between

1996 and 2000 and *Salmonella* were attributed to most fatalities from food-borne diseases (Adak et al., 2005). In this latter report, pork and pig products (ham and bacon) were found to be important sources of food-borne disease, whilst another recent survey has shown that *Salmonella* was isolated from the caeca of 23% of all pigs slaughtered in the UKs' abattoirs (Ivanek et al., 2004). In the USA, about 1 – 4 million cases of the disease and a half million deaths are recorded annually from *S*. Typhimurium, largely as a result of antibiotic resistance (Glynn et al., 1998).

Non-typhoidal of *Salmonella* (NTS) strains, mainly *S.* Typhimurium and *S*. Enteritidis are the most common cause of invasive form of *Salmonella* infection in Malawian children. NTS is mostly common in children aged 1-2 years and relatively less common in children under 4 months old, where they receive maternal antibody. Among children 1-2 years, the amount of immunoglobulins (IgG and IgM) level in children sera were relatively less and resulted in reduce *Salmonella* killing. The study suggests that the cell-mediated immunity plays essential role for killing of intracellular NTS whereas antibody and complement are indispensable for clearance of extracellular bacterial infection (MacLennan et al., 2008). NTS have been shown as a crucial cause of invasive bacteraemia and mortality (20-25%) in Malawian children associated with malnutrition and malaria while with AIDS in adults (Gordon, 2011). NTS is the second most causative agent, after *Streptococci,* of invasive bloodstream infection in Malawian new-borns, causing over 60% mortality (Milledge et al., 2005). By

culturing blood and bone marrow of HIV patients, it was shown that the invasive form of NTS can persist and multiply in cells of bone marrows and blood due to immunological disorders (Gordon et al., 2010). Using blood culture method in children of Nigeria shows that septicaemia caused by *Salmonella* was more prevalent (56%) in children at age between 5 -11 years and followed by 36% aged 1 - 5 years (Ogunleye et al., 2006).

1.2.1 Pathogenesis of *Salmonella* enterica

Salmonella can attach to several sites of the intestinal tract, such as the small intestine, caecum and colon (Darwin and Miller, 1999). Murine studies have suggested that the microfold (M) cells of the Peyer's patches also serve as a major portal of infection (Clark et al., 1994). To invade and survive in cells, *Salmonella* have evolved highly complex biological systems and many genes have now been described to encode diverse virulence factors in *Salmonella*. These genes are clustered, in the bacterial chromosome, to form what are known as *Salmonella* Pathogenicity Islands (SPIs). In *Salmonella* serovars, 23 types of SPIs have been discovered to date (Hayward et al., 2013) while 12 SPIs are particular in *S.* Typhimurium which are SPI-1-6, SPI-9 -14, and SPI-16 (reviewed in López et al., 2012).

In *Salmonella*, SPI-1 (Galán, 1999) and SPI-2 (Hensel, 2000) are the most essential pathogenicity islands needed for the colonisation and invasion of the intestinal barriers. SPI-1 is crucial for the invasion of host cells, such as intestinal epithelial cells and M cells (Steele-Mortimer et al., 2002, Drecktrah et al., 2006). SPI-1 encodes the type three secretion system (TTSS) which translocate a group of bacterial proteins (effectors) into the host cell cytosol (Mirold et al., 2001, Schlumberger and Hardt, 2005). Numerous studies have now indicated that SPI-1 proteins act either together or independently to enhance bacterial invasion. For example, SipA, SipC, SipE, and SipE2 are involved in internalisation of bacteria into the *Salmonella* containing

vacuole (SCV)(Bakowski et al., 2008), whereas SptP antagonise their actions (Humphreys et al., 2009). SipB and SpvB mediate cellular destruction, apoptosis and cytotoxicity (Guiney, 2005) depending on the multiplicity of infection (MOI). When a low MOI (10-20) of *S*. Typhimurium is applied to cell cultures, the infection induces apoptosis, while it results in cytotoxicity if high MOI's are applied (Lindgren et al., 1996). Phagocytic cells engulf the apoptotic cells and this leads to continual dissemination of the infection via transport from the peripheral tissues to deeper, extra-intestinal tissues (Guiney, 2005).

In *S*. Typhimurium, SPI-2 produces about 30 effector proteins which are injected into host cells via the TTSS (Bakowski et al., 2008). The effectors enable survival advantage of the bacteria via the creation and developing of the SCV (reviewed by Figueira and Holden, 2012). The main functions of SPI-2 TTSS effector proteins are to facilitate intra-phagocytic-bacterial persistence via formation of SCV (reviewed by Figueira and Holden, 2012) and failure of *Salmonella* to survive in macrophages is associated with avirulence (Fields et al., 1986). In addition, proteins, such as SseG and SseF, can modulate the outer layers of eukaryotic cells to facilitate their entry. This modulation includes: the biogenic formation of the SCV wall, maturation of the intracellular bacteria, alteration of cellular function and the host immune response (Abrahams et al., 2006). SseG and SseF have also been shown to change the trafficking direction of the SCV and formation of nests adjacent to the Golgi apparatus (Deiwick et al.,

2006, Bakowski et al., 2008). This position allows the bacteria to replicate by the action of the SseG and SseF TTSS proteins (Salcedo and Holden, 2003, Abrahams and Hensel, 2006). Subsequently, the SCV exhibit centrifugal motility after 24 hours of infection mediated by SifA and PipB2. This movement has been shown to allow migration of *S*. Typhimurium from infected Hela cells to uninfected ones within a cell monolayer (Szeto et al., 2009).

S. Typhimurium also contains a two-component regulatory operon known as the PhoP/PhoQ system. This operon is known to control at least 120 different *Salmonella* genes (reviewed by Kato and Groisman, 2008). Most focus has been on the effect of PhoP/PhoQ on SPI-2 genes and it has been shown that transcription of SPI-2 genes is decreased around 100 folds in a PhoP/PhoQ mutant background (Yoon et al., 2009). However, the PhoP/PhoQ operon is also known to control HilE protein, which in turn controls HilA protein (Baxter and Jones, 2015). HilA protein is a critical transcriptional regulator of all SPI-1 genes (Bajaj et al., 1996). Therefore, the PhoP/PhoQ operon has a significant effect on both SPI-1 and SP-2.

1.2.2 Immunity to Salmonellosis

1.2.2.1 Innate Immunity

Innate immunity has a pivotal role in the recognition of pathogens and the induction of the immune response against them (Mogensen, 2009). Once bacteria penetrate the intestinal barrier, a robust cellular response is stimulated which include the activation of innate effector cells such as; macrophages, monocytes, dendritic cells (DCs) and neutrophils (Tam et al., 2008).

Neutrophils are highly phagocytic cells, which are also essential in the innate immune response against *Salmonella*. In murine models of salmonellosis, it has been shown that large numbers of neutrophils migrate into infected tissues for the first 3 days, after which they die and the neutrophil population is replaced by macrophages (Richter-Dahlfors et al., 1997). During *Salmonella* infection, macrophages' inflammasome induces robust pro-inflammatory cytokines by activating caspase-1 pathway such as IL-1β, IL-18 and inducing of cell death (pyroptosis). The released IL-1β and IL-18 recruit neutrophils (Mastroeni et al., 2009, Chen and Schroder, 2013) whereas cell pyroptosis expose *Salmonella* inside pyroptic macrophages to neutrophils (Miao et al., 2010). Neutrophils activate pro-inflammatory cytokines against *Salmonella* but, unlike macrophages, do not elicits to pyroptosis (Chen et al., 2014). Also unlike macrophages where *Salmonella* can survive inside SCVs and propagate the infection, neutrophils granules contain myeloperoxidase which is the main

consumer for ROS production and efficiently kill *Salmonella* (Fenlon and Slauch, 2014).

The role of neutrophils has been investigated in inbred mice, SPRET/Ei originated from *Mus spretus*, which are resistant to *S*. Typhimurium and able to recruit high amounts of neutrophils during infection (Dejager et al., 2010). This study reported that SPRET/Ei mice have a reduced bacterial load when compared to neutropenic litter mates. Another study examined the importance of neutrophils in murine model of salmonellosis. The study showed that neutrophil diffusion throughout the tissues of infected mice was positively correlated with decreased bacterial virulence and the numbers of colony forming units (CFU) recovered (Cheminay et al., 2004). One study has also suggested that there may be a link between tissue neutrophilia and SPI2 in *S*. Typhimurium-infected mice. This study showed that when wild type C57BL/6 mice were infected with *S*. Typhimurium a significantly higher number of neutrophils were found in the Peyer's patches, mesenteric lymph nodes and spleen when compared to SPI2 knock-out (KO) mice infected with *S*. Typhimurium. In this case, increased neutrophilia in wild type mice was associated with decreased bacterial dissemination (Cheminay et al., 2004). Studies using human colorectal carcinoma (T84) monolayers as a model of human intestinal epithelium have shown that neutrophils are recruited by IL-8 (Eckmann et al., 1993) and pathogen-elicited epithelial chemoattractant (PEEC) (McCormick et al., 1998) which are produced when the apical membrane of the T84 cell interacts with *S*.

Typhimurium (Dejager et al., 2010). Following stimulation, neutrophils produce a number of pro-inflammatory cytokines, such as IL-1β, TNF-α and NO (Rydström and Wick, 2007). Therefore, intestinal neutrophilia, following *Salmonella* infection, may serve two purposes. Firstly, it has a direct killing effect, which probably prevents the host being overrun by pathogens, and secondly it increases the concentration of essential cytokines at the site of infection. These cytokines may increase killing pathways in other effector cells or may induce migration of other leukocytes to the site of infection.

Following oral infection of mice by *salmonella*, Peyer's patches (PP) and mesenteric lymph nodes (MLN) are the first tissues invaded by the bacteria (McSorley et al., 2002). In these tissues, neutrophils and monocytes are the primary host cellular defence (day 5 post infection) against the invader while the latter are cells that are more predominant. After *Salmonella* infection, neutrophils attract to the Peyer's Patches of infected mice only in inter-follicular region whilst along with recruited monocytes create inflammatory foci across the infected Peyer's patches (Rydström and Wick, 2009). Monocytes can be distinguished from neutrophils in the mice PP and MLN by high co-stimulatory molecules, CD86 and CD80, while low CD11a expressions (Rydström and Wick, 2007).

Monocytes sorted from blood, Peyer's patches or mesenteric lymph nodes of the infected mice produce higher amount of macrophage inflammatory protein 2-alpha (MIP2-alpha), polymorphonuclear

leukocytes chemoattractant, and Monokine induced by gamma interferon (MIG), chemotactic for T-cell, compared to the control animal. Furthermore, the *ex vivo* cultured-monocytes release further MIP-2 and MIG when reactivate with *Salmonella* (Rydström and Wick, 2009).

Monocytes and macrophages have potential to recognise and respond to various pathogenic determinants such as LPS, flagella, porins, peptidoglycan and lipoprotein (Karlsson et al., 2004). After 40 minutes of *Salmonella*/monocyte/macrophage interaction, the cells undergo morphological changes which include cellular shrinkage, formation of prominent intracytoplasmic vacuoles and membrane blebs, detected by time lapse video microscopy (Monack et al., 2001). The monocytes co-stimulatory molecules along with major histocompatibility complex class II (MHC-II) expressions increase markedly in the tissues during infection and these expressions strength depend on IFN-γ and IL-12 activation. In addition, monocytes and macrophages are a primary source of anti-bacterial mediators such as TNF-α (60 %) and iNOS (85 %) in the MLN of the infected mice with *S*.Typhimurium at day 4 post infection (Rydström and Wick, 2007). Moreover, the cells produce IL-1α, IL-12, IL-15, IL-18 (Eckmann and Kagnoff, 2001) along with IL-6 which is highly associated with the severity and prognosis of sepsis (Spittler et al., 2000).

Murine monocytes can also differentiate into mucosal Dendritic cells (DCs) but not conventional $CD11^{high}$ splenic DCs, which are derived

from committed macrophage/DC precursors in the bone marrow (Varol et al., 2007). Human monocytes can be converted into macrophages (Bender et al., 2004) when co-cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) whereas into DCs if co-culture with GM-CSF and IL-4 (Conti et al., 2008). However, presence of TLR induced IL-6 cytokine in the supernatants of the cultured human monocytes may skew differentiation of monocytes into macrophages (Chomarat et al., 2000) while IL-12P40 and IL-10 incline the differentiation toward DCs (Rotta et al., 2003).

DCs are specialised antigen-presenting cells (APCs) which can phagocyte and convey *Salmonella* through intestinal barriers during infection (Wick, 2003). These cells connect the innate immune system with adaptive immunity, via presentation of antigens, in conjunction with major histocompatibility complexes I and II, (MHC I and MHCII) to T cells (Sundquist et al., 2003). *Salmonella*-induced maturation of DCs results in expression of co-stimulatory molecules, CD86 and CD40, as well as production of cytokines such as interleukin (IL) 6, 12 and tumour necrosis factor alpha (TNF-α) and nitric oxide (NO) *in vivo* and *in vitro* (Pietilä et al., 2005, Zhao et al., 2006). These highly immunogenic DCs then migrate to the lymph nodes where they present antigens to cognate lymphocytes (Zhao et al., 2006), thus initiating the adaptive immune response.

1.2.2.1.1 Pathogen-associated molecular patterns and LPS recognition receptors

1.2.2.1.1.1 Recognition of LPS

The immune response can be initiated by several conserved bacterial components known as pathogen-associated pattern recognition receptors (PAMPS) such as lipoteichoic acid, DNA, peptidoglygans (Jørgensen et al., 2001) and also flagellar proteins such as FliC (McDermott et al., 2000). However, LPS is the most powerful inflammatory agent produced by Gram-negative bacteria and the human immune system is sensitive to LPS concentrations as low as 1 ng/ml (Miyake, 2004). The ability of the immune system to detect LPS is, therefore, critical to resolution of Gram-negative infection and host survival. A number of innate cell receptors bind to the Lipid A moiety of LPS. Mostly studied in monocytes and macrophages, these receptors include; CD14, the macrophage scavenger receptor (SR) and β2 integrins (CD11a/CD18, CD11b/CD18 and CD11c/CD18) (reviewed by Fenton and Golenbock, 1998). Only the CD14 associated LPS-recognition pathway will be discussed further below. CD14 exists in two known forms, a membrane bound glycosylphosphatidylinositol (GPI) anchored form and a soluble form (Ulevitch and Tobias, 1994) both of which bind LPS.

Although LPS can bind directly to the CD14 receptor, this interaction is greatly increased when LPS is bound to the LPS binding protein (LBP) which is present in serum (Tobias et al., 1986, Hailman et al., 1994). LBP is produced by hepatocytes (Ramadori et al., 1990) and intestinal

epithelial cells (Vreugdenhil et al., 1999). The serum concentration of LBP increases significantly during the acute phase response (Tobias et al., 1992) following stimulation of hepatocytes with inflammatory cytokines such as IL-1β and IL-6 (Kirschning et al., 1997). However, since CD14 is a GPI-anchored molecule without a cytoplasmic tail, cellular response to LPS requires further molecular interactions (Lee et al., 1993). These will be considered below.

1.2.2.1.1.2 Toll-like receptors and LPS signalling pathways

Toll-like receptors (TLRs) belong to a group of receptors know as pathogen recognition receptors (PRRs). These receptors detect and/or respond to conserved PAMPS, as stated above. PRRs include those receptors previously discussed, such as CD14, SR and β2 integrins and also nucleotide-binding oligomerization domain (NOD) receptors, or neucleotide binding domain/leucine rich repeat (or NOD-like receptors) (NLRs) and C-lectin receptors (CLRs). However, one of the best studied of all PRRs are the TLR family which consist of ten members in humans and thirteen members in mice (Kumar et al., 2009, Gong et al., 2010). The TLR gene was first discovered in the fruit fly (Anderson et al., 1985) and subsequently found to be involved in immune response to fungi (Lemaitre et al., 1996) and bacteria (Michel et al., 2001). TLR family members can be divided into distinct groups which respond to lipids (TLR 1, 2, 4 and 6), nucleic acids (TLR3, 7, 8 and 9) and proteins (TLR5) (reviewed by De Nardo, 2015). LPS is recognised by TLRs but

which TLR is stimulated depends on the LPS type (Takeuchi et al., 1999) as Gram-negative enteropathogens such as *S.* Minnesota or *Escherichia coli* (*E.* Coli) mainly stimulate TLR4 in human monocytes (Tapping et al., 2000), while LPS from the Gram-negative oral pathogen *Porphyromonas gingivalis* (*P.* Gingivalis) may activate TLR2 or TLR4 (Darveau et al., 2004). However, Foster et al. (2007b) have shown that in the presence of various LPS types, human monocytic THP-1 cells differentiate into macrophages, which express the appropriate TLR receptor for the type of LPS. Therefore, *P.* Gingivalis LPS induced an initial TLR4 expression which was then replaced by primarily TLR2-expressing macrophages, and THP1 cells stimulated with *E.* Coli LPS mainly initiated TLR4-expressing macrophages from the outset, thus for LPS detection, TLR4 is the default TLR.

1.2.2.1.1.3 Activation of TLR4 by LPS and induction of the intracellular signalling cascade

TLR4 activation by LPS requires physical interaction with a membrane bound accessory molecule (MD-2) which links CD14 to TLR4 (da Silva Correia et al., 2001) but some studies have also shown that MD-2 may bind LPS independent of CD14 (Manček et al., 2002, Kobayashi et al., 2006). Point mutations in MD-2 in Chinese Hamster Ovary (CHO) cells abolishes LPS signalling (Schromm et al., 2001) and MD-2 gene knockout mice fail to respond to LPS administration (Nagai et al., 2002) thus showing the critical importance of MD-2.

Dimerization of MD-2/TLR activates the Toll-interleukin 1 (TIR) receptor which is common to all TLRs. This, in turn, activates intracellular adaptor molecules, which induce two different down-stream cascades. The first effect of TIR activation is to induce dimerization of Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) (also known as MAL) together with Myeloid differentiation factor 88 (MyD 88) (reviewed by Pålsson-McDermott and O'Neill, 2004). This MyD88 pathway then recruits Interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK 4 which subsequently results in down-stream activation of the cytokine transcription factor nuclear factor κβ (NF-κβ) and transcription of cytokine genes (reviewed by Kawai and Akira, 2010). The second, MyD88-independent, pathway is initiated when TIR activates TIR domain-containing adaptor-inducing IFN-beta (TRIF) and TRIF-related adaptor molecule (TRAM), which results in activation of transcriptional regulation of cytokines via interferon response factors (IRFs)(Fitzgerald et al., 2003). Thus, the initial detection of Gram-negative LPS (or at least it's lipid A moiety) induces intracellular cascades which leads to activation of different transcription factors that transcribe cytokine genes. These events are shown in figure1.1.

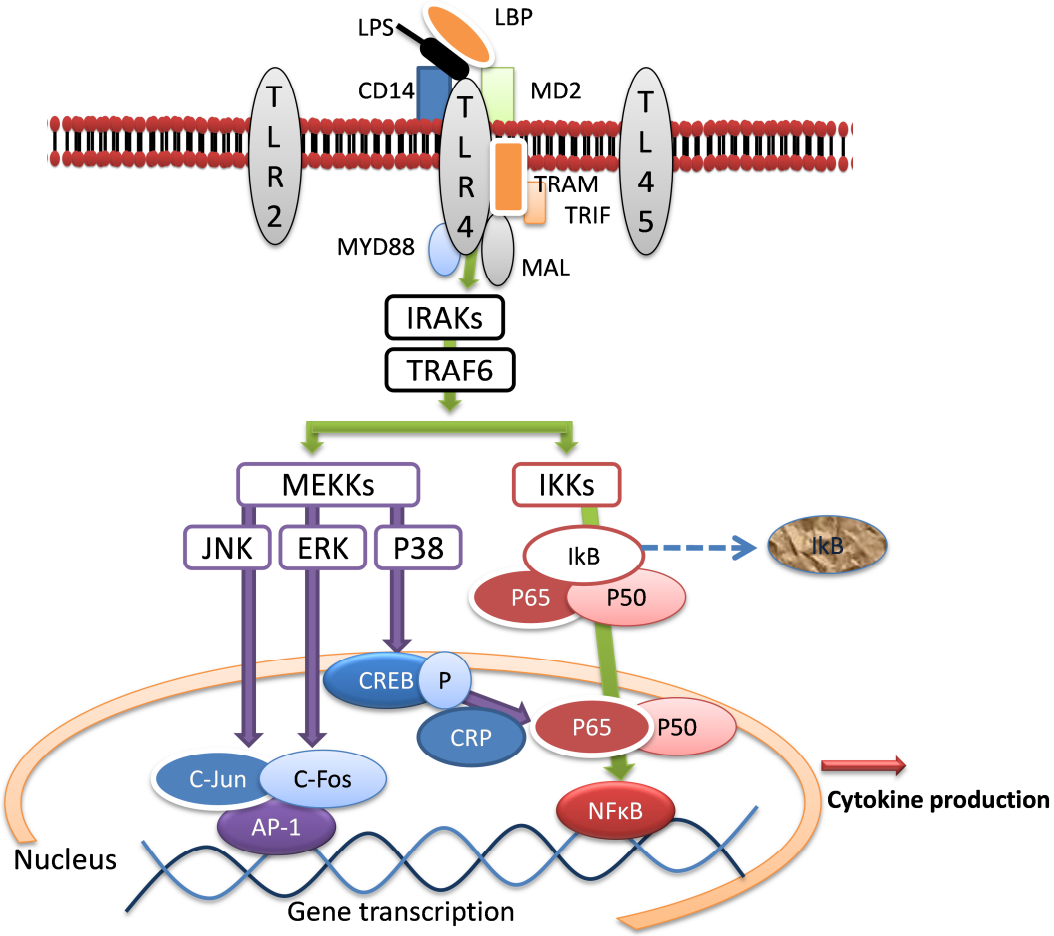

Figure 1.1: Schematic diagram of LPS-TLR4 signalling cascades.
LPS binds with LPS-binding protein (LBP) and then with TLR4, CD14 and myeloid differentiation protein (MD2) receptors, stimulating MyD88-dependent and independent pathway. Through MyD88, LPS induces iκB kinase (IKK) and mitogen activated protein kinase (MAPK) pathways, which subsequently activates transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 and governs the expression of pro-inflammatory cytokines. TIR domain of MyD88 activate IL-1 receptor associated kinases (IRAKs) family then TNF-α receptor-associated factor 6 (TRAF6) which in turn stimulate the inhibitory κB kinase (IKK) and eventually produce nuclear factor κB (NF-κB). LPS, also induce inflammatory mediators via the mitogen-activated protein kinases (MAPKs) down-streaming proteins such as the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase (JNK) and p38. Also, LPS via MyD88-independent can activate INF3 pathway. The figure is Adapted from Lu et al. (2008).

1.2.2.1.1.4 Activation of Protein kinase C (PKC) family in the LPS response

The role of NF-κB in transcribing cytokine genes has been discussed earlier. However, other transcriptional regulators are also activated in response to LPS and other bacterial components. Data from several studies indicate involvement of the PKC family (α, β, δ, ε and ζ) in the regulation of the inflammatory cytokines in response to the LPS. It is been reported that LPS activation also leads to augmentation of PKC (α, δ and ε) in the phosphorylation of down-stream mitogen-activated protein kinase (MAPK), transforming growth factor-*β* (TGF-*β*) activated kinase 1 (TAK1) and ras homolog family member A (RhoA) (Foey and Brennan, 2004). Similarly, it has been suggested that the extracellular signal-regulated kinase (ERK) pathway is induced by MAPK family members (p38, ERK and Jun NH_2-terminal kinase, JNK) in human monocytes stimulated by LPS (Scherle et al., 1998). This study also reported that addition of the ERK inhibitor (U0126) to the monocyte culture media inhibited LPS-induced ERK1 and ERK2 expression and the down-stream production of IL-1β, IL-8 and TNF-α. In a similar study, using murine macrophages, the p38 inhibitor (SB203580) was shown to prevent LPS-induced expression of MAPK and signal transducer and activator of transcription-3 (STAT3) and the down-stream production of IL-10 and TNF-α (Meng et al., 2014).

1.2.2.1.1.5 Involvement of calcium in the LPS response

An increase in internal and external calcium concentration has been reported in murine macrophages (Chang and Parekh, 2004) and also human lung (Zhang et al., 2014) following LPS stimulation. Calcium is an essential component for several biological purposes. Free calcium (Ca^{2+}) concentration inside resting cells is around 10^{-7}M and about 10^4 times lesser than extracellular concentrations. Numerous stimuli mobilise intracellular calcium, either from the extracellular environment (via Ca^{2+} channels) or from intracellular calcium stores (Chin and Means, 2000). In the rat caecal ligation model of sepsis, it has been shown that intracellular Ca^{2+} concentrations are elevated around two folds in smooth muscle cells within the thoracic aorta (compared to controls) (Song et al., 1993) which may have a causal relationship with production of several pro-inflammatory cytokines (Anderson et al., 1999, Thompson et al., 2000). Intracellular Ca^{2+} concentrations were also shown to be elevated in human monocytic THP-1 cells cultured with LPS and this was associated with production of pro-inflammatory cytokines (Wehrhahn et al., 2010). In this latter study, LPS elevated Ca^{2+} in a time-dependent manner but this (and cytokine production) was abolished by removal of extracellular calcium or inhibition of transient receptor potential melastatin 2 (TRPM2), a calcium-permeable cation channel.

In another study, using human bladder epithelial cells, LPS or *E*. Coli induced TLR4/NF-κB-dependent IL-6 production which was also

associated with increased intracellular Ca^{2+}(Song et al., 2007). In this context, Rossol et al. (2012) suggested the increase of external calcium could represent a 'danger signal' and went on to show that Ca^{2+} ions were required for the formation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) and subsequent IL-1β processing. Moreover, a study by Zhou et al. (2006b) has shown a dynamic involvement of Ca^{2+} in transcriptional regulation of inflammatory mediators. In this study, not only was it shown that Ca^{2+} ions were required for LPS-activated, PKC-dependent, NF-κB activation and down-stream transcription of TNF-α and iNOS genes but that this process was inhibited by both intracellular and extracellular Ca^{2+} chelators.

1.2.2.1.1.6 Activation of calmodulin/CAM KII in the LPS response

Many crucial calcium-dependent pathways require a calcium sensing protein called calmodulin (CaM). CaM mediates several essential processes, for instance metabolism, contraction of smooth muscles, apoptosis and inflammatory response (Stevens, 1983). Binding of calcium/CaM modulates many subsequent targets via both protein phosphatases and kinases (Kapiloff et al., 1991). The most important of these is the CaM kinase (CaMK) family, which comprises of multi-functional kinases, CaMK1, 2 and 4 (Colomer and Means, 2007).

Many authors have investigated the effect of CaMK on the immune response following LPS exposure. Hyduk et al. (2007) studied the effect of increased calcium concentration on the activation of leukocyte adhesion molecules in human monocytes. The study showed that increased calcium/CaM augmented phospholipase C (PLC) activity and, as a result, increased activity of the vascular cell adhesion molecule-1 (VCAM-1). In another study, CaMK (II and IV) blockage suppressed induction of ERK, JNK, MAPK and AP-1 pathways following exposure to LPS (Cuschieri et al., 2002). The inhibition abrogated platelet-activating factor (PAF) activity in THP-1 cells (Cuschieri et al., 2005) and reduced (>75%) TNF-α production by human monocytes (Rosengart et al., 2002). Furthermore, inhibition of CaMKIV (by STO609 or KN93) in murine macrophages, following LPS culture, reduced the high-mobility group box-1 (HMGB-1) production (Zhang et al., 2008), which acts as pro-inflammatory mediator and gene regulator in advanced stage of endotoxaemia (Wang et al., 2001, Yang et al., 2004). A later study by Zhang et al. (2011), using Ca^{2+} chelators and CaM inhibitors, confirmed the requirement for Ca^{2+} in LPS-induced HMGB-1 production.

The essential role of CAMKII in the response of macrophages to LPS, was also shown in studies using CaMKII -/- KO mice, which exhibited reduced phagocytic capacity when cultured with *E*. Coli. Also, reduced production of pro-inflammatory cytokines when cultured macrophages were stimulated with LPS, while loss of CAMKII was also associated

with significant increase in survival of mice injected with *E*. Coli, 055:B5, LPS (Racioppi et al., 2012).

There remain many unexplored areas of research regarding the role of calcium/CaM activation in the immune response to Gram-negative bacteria. In particular the effect of viable (whole) bacteria on these pathways, in human immune cells. However, studies reported here show that these are essential molecules in the response to LPS which is an integral component of Gram-negative bacteria.

1.2.2.1.1.7 Involvement of calcium in exocytosis

Increased intracellular calcium concentration is directly correlated with cAMP expression and ultimately increases exocytosis (Branham et al., 2006). Elevated intracellular calcium levels mostly occur via increased phospholipase C (PLC) from the endoplasmic reticulum (ER) or externally via cationic channels (Mattson et al., 2000). Phospholipase C is activated by the G protein coupled receptor (GPCR) or MAPK which induce protein kinase C (PKC) and inositol 1, 4 , 5-trisphosphate (IP3) expression and, as a result, release calcium from the ER (Berridge et al., 2003, Clapham, 2007). Many authors have reported crosstalk between calcium and cAMP. It has been pointed out that cAMP enhances calcium release and this is clearly revealed by using cAMP-stimulating agents such as forskolin and VIP (Hamelink et al., 2002). cAMP is known to be involved in transmitter release in neuronal cells and also hormone excretions in endocrine as well as exocrine cells

(Burgoyne and Clague, 2003, Seino and Shibasaki, 2005). Increased cAMP levels, via PKA protein, enhances phosphorylation of InsP3 (inositol trisphosphate) receptors and subsequently promote calcium release (Bruce et al., 2002, Brown et al., 2004). Protein kinase (PKA) also enhances cAMP level and as a result stimulates calcium-dependent exocytosis (Hatakeyama et al., 2007). Subsequently, cAMP activates Ras-related protein 1(Rap1), a small molecular weight GTPase of the Ras family, and in-turn Rap1 induces calcium dependent exocytosis through Ras-associated protein 3a, Rab3a (Branham et al., 2006, Ferrero et al., 2013).

Calcium exerts its exocytotic effects either directly via C2 domain or indirectly through CaM/CaMKII from EF domain to the soluble *N*-ethylmaleimide sensitive fusion (SNARE) proteins receptors. SNARE proteins involve Syntaxin, Synaptotagmin, Munc13, Rab3-interacting molecules (RIM) and Rabphilin. These proteins regulate internalisation, trafficking of intracellular molecules, membrane fusion and finally perform their exocytosis (Stow et al., 2006). Despite that the precise Rab3 role in immune cell is unknown yet, it is up-regulated in some species immune cells after stimulation. For example, Rab3 is up-regulated in catfish infected with bacteria (Wang et al., 2014), hemocytes (phagocytes in invertebrates) of Chinese mitten crab-stimulated with *Vibrio anguillarum* (Wang et al., 2013) and hemocytes of cotton bollworm challenged with *E.* Coli (Li et al., 2015). In neuron, Rab3 is implicated in vesicle maturation, mobilisation, and membrane fusion at the synaptic junctions (Tanaka et al., 2001).

Rab3 is a small protein of Rab family, its activity governed by a Guanosine triphosphate (GTP) active or Guanosine diphosphate (GDP) inactive form (Yamaguchi et al., 2002). The protein comprises of four isomers (a, b, c and d). Of these, Rab3a mediate calcium-dependent vesicle excretion (Lledo et al., 1994, Tanaka et al., 2001). It is the most predominant Rab protein in murine brain tissue, which underlies its importance in synaptic activity (Von Mollard et al., 1994). GDP dissociation inhibitor (GDI) solubilise Rab3a, detaching it from cell membrane and thus changes Rab3a/GTPase bound state (from GDP to GTP) through the exocytosis process (Garrett et al., 1993). GDI only attaches to Rab3 in a GDP-state and recycles it to cystol (Regazzi et al., 1992).

The positive regulatory function of the Rab3a was confirmed in Rab3a -/- KO mice. Lipovšek et al. (2013) investigated the comparative-morphological changes of the docked vesicles in the Rab3a-deleted murine cells (pituitary melanotroph, adrenal gland chromaffin and endocrine pancreatic cells) with wild type cells. Their findings suggest an essential role for Rab3a in the production and degradation of secretory vesicles required for regulation of exocytosis. Calmodulin (CaM) act similarly to GDI and is enhanced by elevated intracellular calcium concentration. Calcium binds to CaM-Rab3a complexes and activates Rab3a, this interaction is competitively inhibited by GDI (Park et al., 1999, Sidhu et al., 2003a). The interaction between CaM and Rab3a is important since both these proteins take part in exocytosis. In accordance with this, prevention of Rab3a binding to CaM abolishes

exocytosis, while a mutant Rab3a which is unable to adhere to its putative proteins (Rabphilin and RIM), inhibits secretory vesicles mediation of spermal exocytosis (Yunes et al., 2002). Moreover, these findings indicate the inhibitory effect of Rab3a is mediated through its binding with CaM and not via effectors, rabphilin and RIM, proteins (Yunes et al., 2002) as shown in figure 1.2.

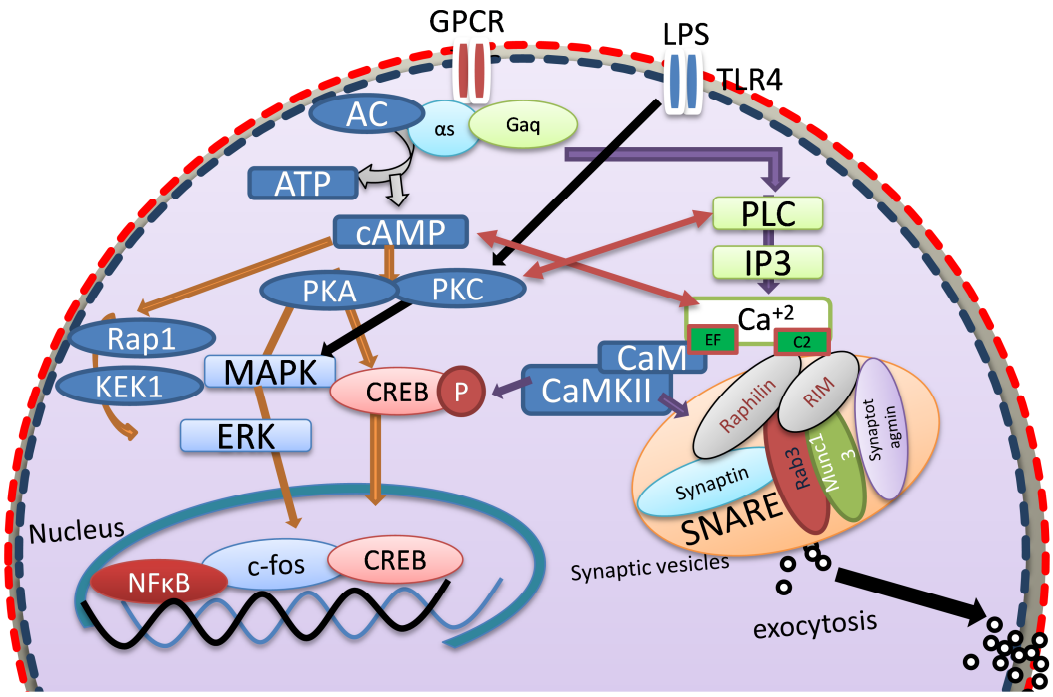

Figure1.2: Intracellular signalling cascades which occur in response of LPS. LPS activates PKC, MAPK then transcription factors of inflammatory cytokines. PKC also may be activated by cAMP or PLC pathway. cAMP is firstly formed by switching adenylate cyclase(AC) to ATP subsequently stimulate RAP1, MEK and then ERK pathway. Calcium is produced from PLC pathway and has a positive correlation with cAMP. Calcium transduces its effects either through calmodulin-CaMKII kinase which in turn up-regulates CREB or trigger SNARE proteins such as Rab3a, Synaptin and Synaptotagmin which mediate move synaptic vesicles toward cell membrane.

1.2.2.1.2 The repertoire and effect of cytokines produced by innate immune cells following LPS/TLR4 interaction

1.2.2.1.2.1 Interleukin 12 (IL-12) family

The IL-12 family of cytokines consists of four, heterodimeric, cytokines (IL-12, IL-23, IL-27 and IL-35) (reviewed by Vignali and Kuchroo, 2012). Two of these (IL-12 and IL-23) will be discussed below.

IL-12p70 is a heterodimeric cytokines produced from the products of two distinct genes which code for IL-12p35 and IL-12p40 respectively (reviewed by Hamza et al., 2010). IL-12 is produced by innate immune cells and in particular APCs, such as those of the myelomonocytic lineage (monocytes, macrophages and dendritic cell). IL-12 production by APCs skews naïve T cell development towards IFN-γ producing Th1 cells (reviewed by Murphy et al., 2000, Wurster et al., 2000) and studies in humans have shown that IL-12R deficiency predisposes to *Salmonella* and *Mycobacterial* infection (de Jong et al., 1998). In murine models of human typhoid disease, IL-12 induced $CD4^+$ T cells and aided to clearance of the bacteria (Price et al., 2007). IL-12 neutralization, by IL-12 antibodies, was also shown to increase the numbers of *S*. Typhimurium C5 recovered from the livers and spleens of infected mice and this was associated with decreased IFN-γ response (Mastroeni et al., 1996).

However, the biology of the IL-12 family is complex, since the IL-12p40 antagonizes the pro-inflammatory effect of IL-12p70, acting as a competitive inhibitor of the IL-12 receptor (reviewed by Cooper and

Khader, 2007). In a murine model of human septic shock, IL-12p40 was secreted significantly more than IL-12p70 and increased the survival rate of mice injected with *E*. Coli LPS (Gillessen et al., 1995). Homodimerization of the IL-12p40 molecule (to form IL-12p80) has also been shown to have an antagonistic effect on IL-12p70 by inhibiting IL-12-dependent, LPS-induced septic shock (Mattner et al., 1997).

The IL-12p40 subunit also dimerizes with another protein (p19) to form the cytokine IL-23, which is active via an IL-23 receptor (Oppmann et al., 2000). IL-23 is a cytokine which activates IL-17A-producing, CD4+ lymphocytes (Th17 lymphocyte) (Weaver et al., 2006, Dong, 2006). IL-17A has been shown to have some effect in clearance of *S*. Enteritidis in infected mice (Schulz et al., 2008). In this latter study, IL-17A was shown to be produced by both classic CD4+, Th17 lymphocytes but also by CD4-, γδ T cells. Humans, with deficiency in the IL-23 axis, are predisposed to systemic bacterial infection (reviewed by Godinez et al., 2011). Although much work is needed to fully ascertain the role of IL-23 in *Salmonella* infection, it certainly appears to be a significant cytokine which further highlights the importance of the IL-12 family in general.

1.2.2.1.2.2 The Interferon family

1.2.2.1.2.2.1 Type I interferons

Type I interferons include Interferon alpha (IFN-α) and beta (IFN-β). The role of these cytokines in intracellular bacterial infection remains unclear. However, a detrimental role for IFN-α and IFN-β have been reported in Mycobacterial infections. For example, addition of IFN-α or IFN-β to human macrophage cultures increased replication of intracellular *M.* Bovis (Bouchonnet et al., 2002) while mice infected with a human tuberculosis isolate showed increased mortality, following intranasal administration of IFN-α/β, which was associated with a decreased Th1 response (Manca et al., 2001). A study by Robinson et al. (2012) has recently reported that murine mortality following infection with S. Typhimurium SL1344 was increased in a type I IFN receptor gene knockout (Ifnar1 -/-) background. Infected macrophages from Ifnar1 -/- mice were also shown to be highly resistant to necroptosis, or programmed necrosis (Galluzzi and Kroemer, 2008).

1.2.2.1.2.2.2 Type II interferon

IFN-γ (the only type II interferon) is essential for the enhancement of cellular killing pathways which mediate clearance of *Salmonella* (Bao et al., 2000). A study by Gordon et al. (2005) explored the role of IFN-γ in the phagocytosis and intracellular killing of *Salmonella* by human monocyte-derived macrophages (MDMs). The study showed that, in

the presence of IFN-γ, MDMs phagocytosed about 30% more *Salmonella* and killed about three times more. A study by Foster et al. (2003) also reported that IFN-γ was essential in the induction of effective reactive oxygen species (ROS) killing pathways required to clear phagocytosed *S*. Typhimurium in murine macrophages (J774 cells). This latter study was also in accordance with that of (Mastroeni et al., 2000) which showed that, following an NO-dependent bacteriostatic effect which gave rise to a population plateau, ROS activation was required to finally decrease *Salmonella* numbers and the study concluded that the ROS role was bacteriostatic rather than killing them. In addition to these studies, a study by Bhosale et al. (2012) has shown that in IFN-γ knockout mice, *Salmonella* survival is increased and this is associated with increased necrotic foci in the liver and spleen.

1.2.2.1.2.3 IL-1 family

The IL-1 family consists of twelve cytokines, of which four (IL-1α, IL-1β, IL-18 and IL-36) will be discussed below. IL-1α, IL-1β and IL-18 are initially produced in an inactive proform which is cleaved into an active form by caspase1 (IL-1β and IL-18) or calpain (IL-1α)(Yazdi et al., 2010), this is not the case for IL-36, which also exists in three isoforms (α, β and γ) all of which bind to the same IL-36 receptor (IL-36R, reviewed in van de Veerdonk et al., 2012). IL-1α, IL-1β and IL-36 are all encoded from a gene cluster on human chromosome 2, in

contrast to IL-18, which is encoded from genes on chromosome 11 (Dinarello et al., 2010).

The synthesis of classical IL-1 cytokines (IL-1α, IL-1β and IL-18) is stimulated by a number of pro-inflammatory mediators including exposure to microbial products such as bacterial LPS or pro-inflammatory cytokines such as TNF-α, IFN-α and IFN-β (reviewed by Barksby et al., 2007). IL-1α and IL-1β are homologous cytokines which bind to the same IL-1R1 receptor (Dinarello, 1996) but IL-1β is secreted while IL-1α is active on the cell membrane (reviewed by Sims and Smith, 2010). IL-1β also tends to be produced predominantly by monocytes and macrophages, whereas IL-1α is produced by a much broader range of cell types (Reviewed by Dinarello, 2009).

Early studies indicated that IL-1α/β have an important role in endotoxaemia since blockade of IL-1R1, by IL-1 receptor antagonist (IL-1RA), was shown to protect mice against Gram-negative sepsis (Ohlsson et al., 1990, Alexander et al., 1991). However, a later study by Fantuzzi et al. (1996) reported that IL-1 $^{-/-}$ gene knockout (KO) mice were not resistant to LPS, thus indicating that IL-1α must be the dominant cytokine in the induction of sepsis. A more recent study by Joosten et al. (2010) has suggested that this is strictly not the case. In this latter study IL-1 $^{-/-}$ KO mice was significantly more susceptible to *E*. Coli and *S*. Typhimurium LPS, possibly due to significantly raised IFN-γ levels. In contrast, IL-1R1 IL-1 $^{-/-}$ KO mice was completely resistant to LPS challenge. This suggests that a very complex

relationship between IL-1α, IL-1β and IL-1R1 exists, in which disturbances in IL-1R1 homeostasis (due to altered relative levels of IL-1α and/or IL-1β) may fundamentally affect LPS resistance or susceptibility.

IL-18 was initially discovered as an LPS-induced protein which caused the secretion of IFN-γ from murine spleen cultures (Nakamura et al., 1989). Production of IL-12 and IL-18 by innate immune cells and, in particular, APCs of the innate immune system are potent signals for the production of IFN-γ by T lymphocytes (Brunda, 1994, Trinchieri, 1994, Okamura et al., 1998). The synergistic effect of IL-12 and IL-18 promotes the formation of a phosphorylated c-Jun/STAT4 complex in T lymphocytes which most efficiently interacts with AP-1 promoter sequence of IFN-γ genes (Nakahira et al., 2001). However, in the absence of IL-12, IL-18 can also promote the proliferation of Th2 subsets (Xu et al., 2000) and the expansion of lymphoid progenitor cells in synergy with IL-7 (Gandhapudi et al., 2015). A study by Dybing et al. (1999) showed that neutralisation of IL-18 decreased IFN-γ production, bacterial clearance and survival of mice challenged with *S*. Typhimurium. Similarly, when *S*. Typhimurium infected mice were treated with recombinant IL-18, increased bacterial clearance was associated with a higher survival rate but this was not observed in IFN-γ $^{-}/^{-}$ KO mice (Mastroeni et al., 1999). A study by (Dreher et al., 2002) reported that SipB protein (SPI2) from *S*. Typhimurium induced IL-18 secretion from human monocyte-derived dendritic cells (MDDCs) but was associated with apoptosis. This study was the first to link

Salmonella TTSS with the IL-1 family and apoptosis and in the following years this phenomenon led to the discovery of the inflammasome (discussed in more detail at the end of this section).

IL-36 is a cytokine which has three isoforms (α, β, γ). This cytokine is one of a group of novel IL-1 family members discovered as a result of the human genome project (reviewed by Sims and Smith, 2010). IL-36α is released in parallel with mature IL-1β from murine bone marrow derived macrophages stimulated with LPS from *E*. Coli (Martin et al., 2009). However, in human myelomonocytic cells the receptor for the IL-36 isoforms (IL-36R) is only expressed by plasmacytoid DCs and MDDCs (Mutamba et al., 2012). This latter study has shown that both IL-36β and IL-36γ are potent maturation signals for human MDDCs which then induce Th1 proliferation. A recent study from the same group has also shown that IL-36α and IL-36β promote increased surface expression of CD14, iC3b opsonic receptor CD11C by human MDDCs and subsequent increased iC3b binding (Higgins et al., 2015). These events were synergistically increased by IFN-γ. Furthermore, IL-36β and IL-36γ increased phagocytosis of *Salmonella* but this was not synergistically increased by IFN-γ. Nevertheless, the study does show an important role for novel IL-1 family members in innate cell response to *Salmonella*.

Inflammasomes are multiprotein complexes which are formed in the cytosol of innate immune cells in response to inflammatory signals. Three of these require nucleotide binding domain/leucine rich repeat

(NLR), these are NLR protein 1 (NLRP1); NLRP3 and NLR-CARD domain 4 (NLRC4). A fourth inflammasome requires absent in melanoma 2 (AIM2) whilst the fifth inflammasome is non-canonical type which contains none of these proteins (reviewed by Shin and Brodsky, 2015)(Fig 1.3). Of the NLR-containing inflammasomes, NLRC4 recognises bacterial flagellin and TTSS proteins (Molofsky et al., 2006, Sutterwala et al., 2007, Zhao et al., 2011). The non-canonical from recognises cytosolic LPS and produces caspase 11 (Broz and Monack, 2013, Rathinam et al., 2012), whereas the other inflammasomes produce caspase 1 which induces programmed necrosis (pyroptosis) and/or processing of mature IL-1β and IL-18 (reviewed by Shin and Brodsky, 2015). Therefore, IL-1 cytokines are critical to the formation and effect of these inflammasomes.

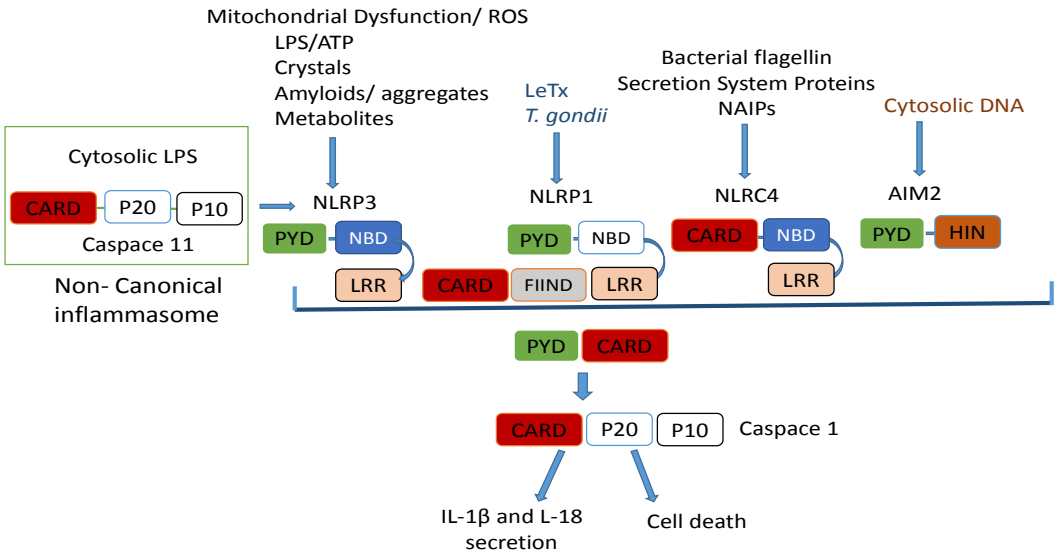

Figure 1.3: Effect of various stimuli on the induction of inflammasomes and production of IL-1β and IL-18
(Adapted from Shin and Brodsky, 2015).

1.2.2.1.2.4 TNF-α superfamily

The TNF-α family consists of more than twenty ligands (and receptors) and is therefore termed a 'superfamily.' Only one member of this superfamily (TNF-α) will be discussed below. TNF-α is a powerful pro-inflammatory cytokine employing pleiotropic action on diverse cell types and is fundamental in the pathogenesis of several inflammatory and non-inflammatory diseases (Bradley, 2008). TNF-α has two forms, transmembrane and soluble TNF-α. Transmembrane TNF-α (tTNF-α) is the originator of soluble TNF-α (sTNF-α) and found on activated immune cells. The biological activity of tTNF-α require cell to cell physical connection whereas the activity of sTNF-α occurs in distant areas from TNF-α generating cells (Reviewed in Horiuchi et al., 2010). In support of this, a reduced immune response and mortality was reported in TNF-α receptor 1 (TNFR1) or TNF-α receptor 2 KO (TNFR1^{-}/$^{-}$ TNFR2 $^{-}$/$^{-}$) mice in which caecal contents were injected intraperitoneally. In contrast wild type mice went on to contract polymicrobial sepsis (Secher et al., 2009).

TNF-α has several biological activities ranging from cell proliferation, differentiation and cell death and is also an essential cytokine for the induction of cellular immune responses (Wallach et al., 1999). TNF-α mediate its biological activities through two receptors TNFR1 and TNFR2. TNF1 is constitutively expressed and can be distributed almost all nucleated cells whereas TNFR2 is inducible and found in T lymphocytes and myeloid lineages (Hijdra et al., 2012). Induction of

the intracellular signalling cascades by TNF-α results in stimulation of NF-kB and many MAPK-induced pathways such as Jun N-terminal kinase (JNK), Extracellular signal-regulated kinases (ERK) and P38 which stimulates production and release of many cytokines such as IL-6 (Reviewed in Berghe et al., 2000). Following LPS exposure, interferon-γ by resident or recruited Th1 cells induces the production of several macrophage mediators, including TNF-α (Reviewed in Spooner et al., 1992). TNF-α is a principal mediator of septic shock (Spooner et al., 1992). Indeed, administration of recombinant TNF-α into rats resulted in the symptoms of sepsis (changes in endothelial permeability, coagulation, metabolic acidosis, hypotension and multiple organ dysfunction) followed by respiratory failure and death (Tracey et al., 1986). These physiological effects can be abolished by suppression of TNF-α (Matot and Sprung, 2001).

1.2.2.1.2.5 IL-10

The IL-10 family comprises of six members (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) (reviewed by Sabat, 2010) but only one of these (IL-10) will be discussed below. Interleukin-10 (IL-10) is a pleiotropic cytokine that can modulate functions of various cell types. The cytokine is produced by monocyte/macrophages, dendritic cells, B and T cells (Moore et al., 2001). IL-10 is an immunomodulatory cytokine which supresses the production of various inflammatory mediators by cells during infection. The cytokine regulate $CD4^+$ T cells function through restricting of generation of IL-2, TNF-α and IFN-γ (Reviewed in Couper et al., 2008).

In *vivo* studies have reported that serious or lethal immunopathology occurred in IL-10 $^-/^-$KO mice infected with *Toxoplasma gondii* (Wilson et al., 2005) or *Trypanosoma* (Hunter et al., 1997) compared to wild type controls. The production of pro-inflammatory cytokines and also anti-bacterial astrocytic function was also elevated in IL-10 $^-/^-$KO infected with *Toxoplasma gondii* mice compared to wild animals and this was associated with increased macrophages and $CD4^+$ T cells in murine brains (Wilson et al., 2005). IL-10 modulate activities of natural killer (NK) and T cells indirectly via down-regulation of MHCII and costimulatory molecule (B7-1/B7-2) on monocyte/macrophage cells and subsequently reduce the production of some pro-inflammatory cytokines and chemokines (reviewed in Moore et al., 2001). Similarly, absence of IL-10 resulted in elevation of IL-1β, IL-12

and TNF-α in the culture of human monocytes exposed to LPS (Shin et al., 1999). Likewise, IL-10/IL-10 receptor ligation can modulate pro-inflammatory cytokine production in monocytes via phosphorylation of receptor-associated Janus tyrosine kinases (JAK1) and subsequently activate STAT3 which in turn reduces inflammatory mediators in LPS-stimulated human monocytes (Donnelly et al., 1999). Latifi et al. (2002) reported that IL-10 is able to improve survival of endotoxaemic mice induced by caecal ligation and puncture. The study showed that IL-6 and TNF-α concentration in mouse serum was over 15 folds higher also a significantly lower survival rate was recorded in the IL-10 $^{-}/^{-}$KO group when compared to the control group.

1.2.2.1 Adaptive Immunity

The importance of innate immune cell-killing pathways for clearance of *Salmonella* from the host has been discussed above. For *Salmonella* clearance, the adaptive immune response needs to be stimulated which includes T (CD4 and CD8) and B lymphocytes (Mittrücker and Kaufmann, 2000). *Salmonella* exploit the microfold (M) cells and access into host epithelial wall, which are specialised antigen sampling cells they uptake *Salmonella* then deliver them to underlying phagocytic cells (Jepson and Clark, 2001). Dendritic cells appear after about 3 hours in the Peyer's patches of mice orally challenged with *Salmonella* (McSorley et al., 2002). Dendritic cells (DC) connect innate to adaptive immunity (Fig 1.4) via capture and presentation of bacterial antigens to naïve $CD4^+$ T cells (Wick, 2003). The immunogenic DCs induce $CD4^+$ T cells to secrete IL-2 (T cell mitogen) and promotes the migration of CD4+ lymphocytes to B cell follicles for production of antigen-specific antibody (Jenkins et al., 2001).

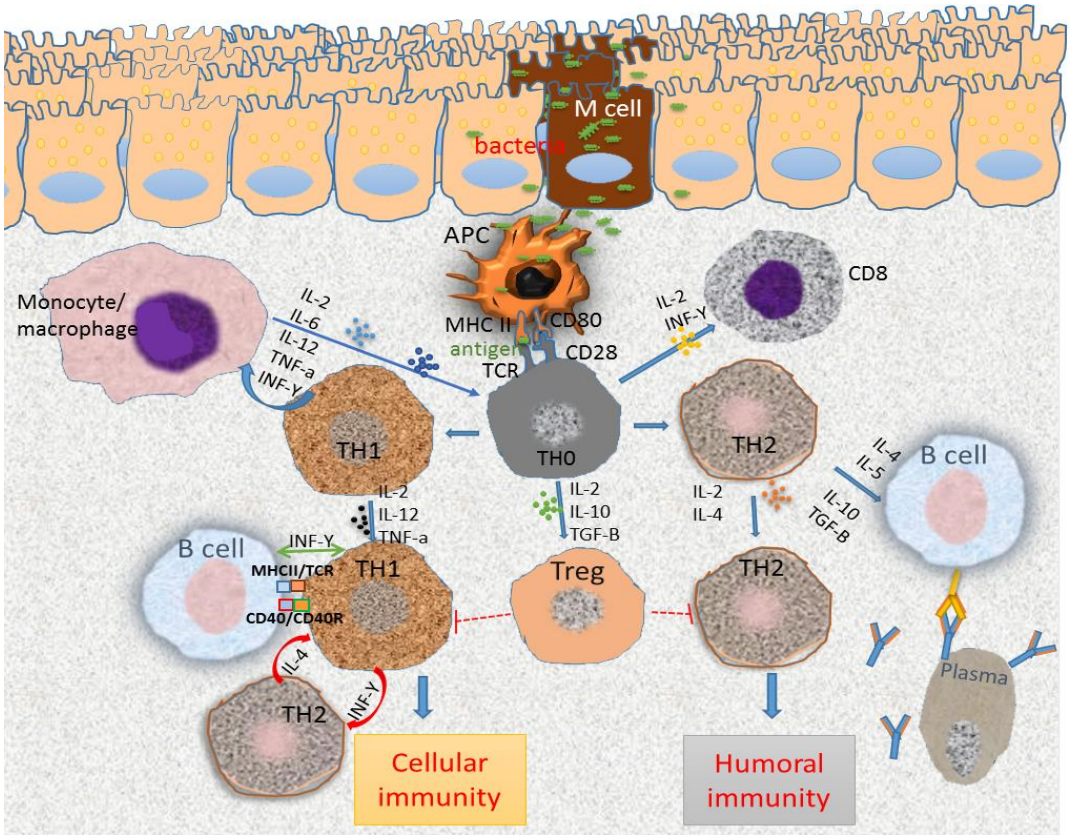

Figure 1.4: Overview of cell-mediated pathway following bacterial recognition.
APCs recognise phagocyte then present bacterial peptide via MHC II to naïve CD4 T cell. Naïve CD4, in turn, proliferates and differentiates CD8, TH1, TH2 or regulatory (Treg) T cells depending on the secreted cytokine. Subsequently, TH1 proliferate, differentiate more TH1 cells, and produce certain cytokine resulting in macrophage / monocyte stimulation. In the same manner, TH2 precede more cells and active B cells which is responsible for antigen-specific antibody production. The figure is adapted from Zhu and Paul (2008).

Upon antigen presentation by APCs, the naïve CD4 T cells (T helper cell type zero, or Th0 cells) are stimulated via interaction of their receptors (TCR) with antigen presented in conjunction with MHCII on the APC surface. The naïve CD4 T cells then differentiate into effector subsets such as Th1, Th2 and Th17 cells. The differentiation of Th0 cells into Th1 or Th2 subsets depends upon several factors such as the

concentration of the antigenic peptide being presented the nature of pathogen and the cytokines pattern produced by the APC (Del Prete, 1998, Janeway et al., 2001). Higher quantities of antigenic-peptide presented to TCR may direct CD4 T cells response to differentiate into Th1 not Th2 (Janeway et al., 2001). Cytokine type also determines the nature of the CD4 T cells response. For example, in IFN-γ and IL-2 cultured with human CD4 T cells derive cytokine production toward TH1 while IFN-γ with IL-4 skews the produced cytokine toward TH2 (Torres et al., 2004).

Following *Salmonella* infection, CD4 T cells rapidly differentiate into Th1. Activated Th1 cells enhance killing of *Salmonella* by producing IFN-γ (Mittrücker and Kaufmann, 2000) and TNF-α which are essential for the host immune response against *Salmonella* infection (Dharmana et al., 2001). After *Salmonella* infection, Th1 cells proliferate and, after about 2-3 weeks, Th1 cells represent about 50% of the total CD4 T cell population (Srinivasan et al., 2004). Th1 cells secrete cytokines such as IFN-γ, TNF-α and IL-2 which are essential in the activation of innate cells (Romagnani, 1999), while production of IL-2 and IFN-γ are important in the activation of CD8+ lymphocytes (Janeway et al., 2001).

After an initial Th1 response which activates innate immunity, a Th2 response is produced which stimulates antibody production. Th2 cytokines such as IL-4 (Okahashi et al., 1996) and IL-5 (Beagley et al., 1988) are important mediators for mucosal immunoglobulin A (IgA)

which are crucial for mucosal defence against intracellular pathogen. IL-4 is an important TH2 cytokine which modulate immune response during *Salmonella* infection through suppression of Th1 cytokine response (Liu et al., 2010). IL-4 cytokine together with granulocyte macrophage colony stimulating factor (GM-CSF) generate DCs from monocytes (Anguille et al., 2009, Harris et al., 2010). The immune response of monocyte bias toward TH1 or TH17 when the co-cultured with GM-CSF and IL-15 (Harris, 2011). The main function of IL-15 is to maturate and regulate function of NK cells during early immune response against *Salmonella* (Ashkar et al., 2009). GM-CSF roles in *Salmonella* infection have been revealed in GM-CSF deleted mice. The deleted GM-CSF reduced elimination of *Salmonella* by suppression in pro-inflammatory cytokines such as IL-12, IL-18 and TNF-α (Coon et al., 2009). Th2 also induce B cells response for production of some classes of immunoglobulin such as IgG, IgA and IgE (Street and Mosmann, 1991).

Th17 CD4 T cells are involved in protection against infectious organisms (McGeachy and McSorley, 2012) but are also associated with the development of autoimmune diseases (Gaffen et al., 2011, Iwakura et al., 2011). Furthermore, the Th17 producing cytokine such as IL-17 induce granulopoiesis and subsequently stimulate proliferation and attraction of neutrophils at infection areas (Cua and Tato, 2010). Keestra et al. (2011) investigated that intestinal Th17 production in a murine model is dependent on MyD88 and IL-1β receptor and occur after 24h post- *Salmonella* infection. In this study, IL-17A was shown

to be produced by 3 distinct intestinal T cell populations. These were; δγ T cells, Th17 cells and a populations of CD4-/CD8- lymphocytes. Although the absence of IL-17 signalling was associated with reduced chemokine activity, it had no effect on *Salmonella*-associated intestinal lesions. Therefore, it appears that IL-17 may have some importance during the early phase of salmonellosis but it is clear that the cell types which produce IL-17 and the mechanistic effect of this cytokine during salmonellosis needs much greater study.

The role of *Salmonella*-specific memory T cells in mice has been explored by Kirby et al. (2004). In this study, memory T cells were retained in the blood and lymphoid tissues and memory T cells of both CD4 and CD8, which remain over 6 months, produced TNF-α and IFN-γ rapidly following secondary exposure to *Salmonella* antigens.

Luu et al. (2006) reported that CD8+ lymphocytes are actually very slow to react to *Salmonella* antigens, with a peak in CD8+ effector cell concentrations taking around 3 weeks to occur. Another study has shown that the potency of the CD8+ response to *Salmonella* depends upon the intra-phagocytic amount of bacteria. For example, in a study comparing the burden of wild type *S*. Typhimurium SL1344 in mice with the burden in mice infected with *S*. Typhimurium SL1344 harbouring SPI-2 mutations, it was shown that decreased burden due to SPI-2 mutation was associated with decreased CD8+ proliferation (Sad et al., 2008). However, this study also showed that although SPI-2 mutants induced lower numbers of proliferating CD8+ lymphocytes,

the cells were more functional (greater expression of CD62L and IL-7Rα) than was measured in CD8+ lymphocytes recovered from wild type infected mice.

B lymphocytes are also critical in the adaptive immune response to *Salmonella* infection (Mittrücker and Kaufmann, 2000). B lymphocytes can present *Salmonella* antigen via MHCII to activate Th2 lymphocytes (Sproul et al., 2000). The fully activated B lymphocytes also require interaction between CD40-CD40 ligands and CD80/CD86 with CD28 on T cells, to activate Th2 lymphocytes (Wykes, 2003, Rau et al., 2009) (Wykes, 2003, Le Bon et al., 2006, Rau et al., 2009). Consequently, B cells either initiate memory B cells or proliferate and differentiate into plasma cells which commence immunoglobulin class-switching and the production of relevant antibody classes against *Salmonella* (Souwer et al., 2009).

1.3 Vasoactive Intestinal Peptide

1.3.1 Introduction

Vasoactive intestinal peptide (VIP) is a pleiotropic peptide consisting of 28 amino acids which was firstly isolated by Said and Mutt from porcine small intestines (Said and Mutt, 1970). The peptide can be isolated from very diverse tissues. For example, it is highly prevalent in central nervous system tissues such as hypothalamus, thalamus, brainstem, cerebral cortex and cerebral neurons (Joo et al., 2004) but it is also found in the small intestine, stomach, liver (Usdin et al., 1994) and reproductive organs (Gabbay-Benziv et al., 2012).

VIP belongs to the secretin family of peptides, which modulate the action of G-protein coupled receptors (GPCR). The family also comprises of peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), pituitary adenylate cyclase activating polypeptide (PACAP), secretin, glucagon, glucagon like peptide, parathyroid growth factor and growth factor releasing hormones (Laburthe et al., 2007). The sequence of VIP amino acid is homologous to the other family peptides. It shares 68% homology to PACAP (Vaudry et al., 2000), 50 % to PHM and PHI and 33 % to secretin (Fahrenkrug, 2010) as shown in table 1.1. VIP and PACAP exhibit identical affinity to VIP Receptors 1 and 2 (VPAC1/VPAC2), whereas PACAP has 1000 folds greater affinity to PAC1 (PACAP Receptor1) than VIP (Dvoráková, 2005).

The amino acid sequence of VIP has been much conserved in the evolutionary radiation of terrestrial vertebrates (as shown in Table 1.1).

Peptides	Amino acids						Homology With VIP %
VIP	**HSDAV**	**FTDNY**	**TRLRK**	**QMAVK**	**KYLNS**	**ILN**	
PACAP	**HSD**GI	**FTD**S**Y**	S**R**Y**RK**	**QMAVK**	**KYL**AA	V**L**	**68**
PHI	**H**A**D**G**V**	**FT**SDF	S**RL**LG	**Q**LSA**K**	**KYL**ES	**L**I	**50**
Secretin	**HSD**GT	**FT**SEL	S**RLR**D	SARLQ	RL**L**QG	**L**V	**33**

Table1.1: Sequences and homology of amino acids in the VIP and some secretin family-peptides structure.
The coloured and underlined amino acids refer to the shared amino acids between VIP and other family peptides (adapted from Igarashi et al., 2011).

The structure is homologous; originate from the same ancestor or species, in all mammals except the guinea pig which bear retains 86% homology to other mammals, with only four amino acid substitutions. Chicken, alligator and frog have identical, exactly the same sequence and structure, VIP but are different from mammalian VIP (Table 1.2) by substitution of 5 amino acids (Reviewed in Smalley et al., 2009, Foster, 2012). Also, the amino acid structure of VIP in some fishes, such as rainbow trout and bowfin, is identical to each and have only 4

amino acid substitutions to that of the chicken (Wang and Conlon, 1995). In addition, the homology of VIP amino acid sequences across different phyla is associated to physiological studies. For example, guinea pigs and cod differ by only 5 amino acid substitutions and both are equipotent in their ability to release amylase from pancreatic acini cells of guinea pig (Thorndyke et al., 1989).

Species	Amino acids					
Human, cow, horse, pig, dog, cat, rat and mouse	HSDAV	FTDNY	TRLRK	QMAVK	KYLNS	ILN
Guinea and pig	HSDAL	FTDTY	TRLRK	QMAVK	KYLNS	VLN
Chicken, Alligator and Frog	HSDAV	FTDNY	SRFRK	QMAVK	KYLNS	VLT
Cod	HSDAV	FTDNY	SRFRK	QMAAK	KYLNS	VLT

Table1.2: Sequences and homology of amino acids in the VIP of different species.
The coloured and underlined amino acids refer to the shared amino acids between VIP and in other species (adapted from Smalley et al., 2009).

VIPergic neurones are found throughout the body, including lymphoid tissues (Bellinger et al., 1996) and the peptide is also secreted from immune cells. These include murine mast cells (Wershil et al., 1993); Th2 lymphocytes (Delgado and Ganea, 2001a); thymocytes, and B cells (Abad et al., 2002). This will be discussed more fully below

1.3.2 The effect of VIP on immune cells

Numerous studies since the early 1990's have shown a generalised immunosuppressive effect of VIP on the production of inflammatory immune mediators and molecules, mostly in cells from the myelomonocytic lineage.

In macrophages, it has been shown that VIP exerts several functions during infection. VIP inhibits generation of pro-inflammatory, TNF-α IL-1β and IL-6, cytokines via inhibition of NF-kB (Delgado et al., 1998). In another study by Delgado et al. (1999f) it was shown that VIP and PACAP differentially regulate expression of co-stimulatory molecules on the surface of murine peritoneal macrophages, depending on the state of activation of the cell. Therefore, VIP and PACAP enhanced expression of B7.2 (CD86) on the surface of naïve macrophages but no effect on B7.1 (CD80) was detected, by flow cytometry analysis. The peptide down-regulated both B7.1 and B7.2 on the surface of murine macrophages stimulated with LPS, IFN-γ or mouse anti CD40 antibody. VIP/PACAP down-regulated the expression of B7.1/B7.2 on the surface of murine macrophages induced by LPS and prevents their co-stimulatory activity. VIP has also been shown to inhibit many pro-inflammatory mediators produced by monocytes. VIP-induced inhibition of NF-κB, significantly decreased IL-8 production by THP-1 cells cultured with *E*. Coli LPS (Delgado and Ganea, 2003) which could have a down-stream inhibitory effect on neutrophil chemotaxis in response to Gram-negative infection *in vivo*. In other studies using

THP-1 cell, VIP inhibited nuclear translocation of both c-Jun and NF-κB in response to LPS from *P*. Gingivalis and this subsequently reduced TNF-α production (Foster et al., 2005a). Using the THP-1 human monocyte model, the same group also showed that VIP inhibited translocation of the family transcription factor PU.1, in response to LPS from both *P*. Gingivalis and *E*. Coli, which may have reduced the surface expression of TLR2 and TLR4 (Foster et al., 2007b). Thus, VIP can inhibit the early detection of different types of LPS from Gram-negative bacteria (oral and enteric pathogens). In another study, (Foster et al., 2007a) investigated the effect of VIP on production of IL-18 and IL-18BPa, IL-18 inhibitor binding protein A, by THP-1 cells exposed to LPS from *P*. Gingivalis and *E*. Coli. In this latter study, it was shown that although VIP prevented LPS-induced IL-18 production, it also inhibited up-regulation of IL-18BPa. Therefore, this study showed that VIP acted independently of IL-18BPa, with regard to IL-18 inhibition, and may act as a 'master' suppressor.

VIP has a differential effect on DC immunogenicity depending on the maturation status of the DCs (Chorny et al., 2006). In the case of immature murine bone marrow derived dendritic cells (BMDDCs), cultured with VIP, B7.2 expression was increased and in DC/T cell cultures, proliferation of Th2 lymphocytes occurred. In contrast to this, in DCs stimulated with LPS, the addition of VIP reduced expression of B7.1 and B7.2 and inhibited T cell proliferation, while the addition of VIP to cultures of differentiating BMDDCs induced a tolerogenic phenotype (low CD40, B7.1, B7.2 and low production of pro-

inflammatory cytokines) which induced proliferation of suppressor T cells *in vitro* and *in vivo*.

VIP also reduces the Th1 response and promotes Th1/Th2 balance through suppression of IL-12 suppression production by APCs (Delgado et al., 2004). The mechanism for this is believed to be due to the induction of increased production of protein tyrosine phosphatase, an enzyme that eliminate phosphate group from phosphorylated residues on proteins (Liu et al., 2007). Furthermore, VIP inhibits the ability of both CD4 and CD8 T cells to induce apoptosis and cytotoxicity via down-regulation of membrane bound Fas ligand (Ganea and Delgado, 2002).

In serum, VIP has very short half-life, less than 1 minute, due to action of proteolytic enzymes (proteases), particularly trypsin (Gozes et al., 1999, Refai et al., 1999). Therefore, some studies have used VIP incorporation into liposomes as a means to increase its biological stability (Sethi et al., 2005, Stark et al., 2008). A study by Refai et al. (1999) has reported an 8 folds increase in the half-life of VIP can be achieved by liposome encapsulation, but it should be noted that the polymer make-up of these microspheres will impact on half-life and the biological availability of VIP to the host. The normal range of VIP in serum varies from 3 – 30 pmol/l (Becker et al., 2002). This concentration may increase dramatically in individuals with a pancreatic neuroendocrine tumour (VIP-Oma), a very rare pancreatic endocrine neoplasia that secretes VIP, in which serum VIP

concentration may reach 300–1500 pmol/l. The clinical effect of this is hypokalaemia, increase acidic metabolism and profuse watery diarrhoea which may result from the inhibitory effect of VIP on intestinal smooth muscle contraction (Ghaferi et al., 2008).

1.3.3 Vasoactive intestinal peptide receptors

The biological action of VIP occurs via ligation of three receptors. These are vasoactive intestinal peptide receptor 1 (VPAC1), VPAC2 and pituitary adenylate-cyclase activating peptide receptor 1 (PAC1). These receptors belong to the class II family of G-coupled protein receptors (GPCRs) and both VPAC1 and VPAC2 with a similar affinity bind to VIP and PACAP (reviewed by Smalley et al., 2009). Each receptor will be discussed in more detail below.

1.3.3.1 VPAC1

VPAC1 was first cloned from a cDNA library of rat lung (Ishihara et al., 1992). It is predominantly expressed in the brainstem, cerebral cortex, cerebral nuclei, hippocampus, thalamus and hypothalamus of rat brain (Joo et al., 2004). The receptor is also distributed in liver, lung and intestine (Usdin et al., 1994), thyroid, prostate glands, kidney as well as pancreas (Reubi, 2000).

VPAC1 is the most highly expressed VIP receptor in resting immune cells. It is constitutively expressed by resting human monocytes (Lara-

Marquez et al., 2001, El Zein et al., 2006), $CD4^+$, $CD4^-CD8^-$ and $CD8^+$ T lymphocytes subsets in splenic and intestinal lamina propria (Qian et al., 2001). VPAC1 is also constitutively express in murine T cells and macrophages (Delgado et al., 1998).

The anti-inflammatory action of VIP on LPS-stimulated murine macrophages has been shown to occur via VPAC1 (Delgado et al., 2004) and it is via VPAC1/VIP interaction that mice are protected from lethal LPS administration, as shown by studies using specific VPAC1 and VPAC2 agonists (Delgado et al., 1999a, Delgado et al., 1999b, Delgado et al., 2000a). Other studies have also suggested that the immunosuppressive effect of VIP is mediated via VPAC1 rather than VPAC2. For example, VIP/VPAC1 suppresses pro-inflammatory cytokine such as TNF-α, nitric oxide, IL-6 and IL-12, produced by LPS-stimulated murine macrophages (reviewed by Leceta et al., 2000), while Kim et al. (2000) have also shown that VIP-induced inhibition of TNF-α production by rat microglial cells, cultured with LPS from *E*. Coli 055:B5, also occurs via VPAC1 (and PAC1) but not VPAC2.

VIP/VPAC1 ligation may induce its inhibitory effect either in a cAMP-dependent or independent pathway. In the cAMP-dependent pathway, VIP enhances AC, phosphokinase A (PKA), and then cAMP response element binding protein (CREB). Subsequently, CREB interacts with CREB binding protein (CBP) and ultimately decreases activity of NF-κB by block it's binding to CBP. The inhibitory effect of VIP/VPAC1 also may be cAMP-independent. VIP supress inhibitory κB kinase (IκK)

function which in turn enhance IκB/NF-κB stability, thus preventing nuclear translocation of NF-κB (Reviewed in Smalley et al., 2009).

Why the inhibitory effect of VIP occurs via VPAC1 rather than VPAC2 is scientifically interesting and has relevance in the development of novel anti-inflammatory therapies. As discussed early, VPAC1 is expressed constitutively by human monocytes and murine macrophages, whereas VPAC2 is not. This would obviously suggest that VPAC1 is immediately available to VIP and studies have shown that VPAC2 requires about 12 hrs for maximal expression (following stimulation of cell) while VPAC1 binding also produces greater concentrations of cAMP and intracellular Ca^{2+} concentration (Lara-Marquez et al., 2001, Delgado et al., 2004). All of which have significant effect on pathways which exert biological effect due to VIP (Delgado et al., 2004, Langer and Robberecht, 2005, Gonzalez-Rey et al., 2007b).

An interesting, and more recent, study by (Storka et al., 2013) reported that when human volunteers were injected with a bolus containing 2 ng/ml LPS, VPAC1 mRNA expression (but not VPAC2 or PAC1 expression) was increased in peripheral leukocytes and this was associated with increased concentration of VIP in plasma. Although this study does not show any causal relationship between reduction in LPS-induced cytokine production and VPAC1/VIP interaction in humans, it does at least provide some anecdotal evidence to suggest that a similar effect to that shown in murine models of endotoxaemia may also occur in human patients.

1.3.3.2 VPAC2

VPAC2 receptor was cloned from rat olfactory bulb, cDNA library (Lutz et al., 1993) and has also been isolated from mouse (Inagaki et al., 1994), guinea pig (Zhou et al., 2006a) and human (Harmar et al., 1998) tissues. VPAC2 is extensively distributed throughout the bodily tissues. It is highly concentrated in the hippocampus, hypothalamus, brainstem, cerebral and cerebellar cortex of rat brain (Joo et al., 2004). It has been isolated in the smooth muscles of human airways (Groneberg et al., 2001) and the adrenal glands, kidney, pancreas, colon, thyroid glands and retina in mice (Harmar et al., 2004).

In brain, VIP/VPAC2 plays a wide variety of functions range from governing circadian rhythms, metabolism and eating behaviour (Bechtold et al., 2008, Hughes et al., 2011), along with protection from excitoxic white matter damage in mice (Rangon et al., 2005). Additionally, it has been suggested that the receptor may have an impact on schizophrenia (Levinson et al., 2011) and multiple sclerosis (Sun et al., 2006). Using a recombinant protein antagonist of VPAC2 (rMBAY), Yu et al. (2007) have shown that VPAC2 is involved in insulin release and reduced blood glucose in mice. While in human studies, Groneberg et al. (2001) have shown that VPAC2 mediated vasodilation and bronchodilation.

Expression of VPAC2 by immune cells varies according to cell type. It is exclusively expressed in human mast cells (Kulka et al., 2008), raw 264.7 (Murine macrophage cell line). Herrera et al. (2009) have shown

that VPAC2 expression is regulated via TLR2 and TLR4 ligands in murine peritoneal macrophages, which would explain why VPAC2 is not expressed constitutively by these cells (Delgado et al., 1999d). Overexpression of VPAC2 in lymphocytes (isolated from transgenic C57BL/6 mice which overexpress Th2 cells) was shown to be involved in enhanced levels of IgG, IgE and eosinophils in murine blood, while in $CD4^+$ T cells, overexpression of VPAC2 increased IL-4 and IL-5 and reduced IFN-γ following TCR ligation, indicative of an allergic state (VOICE et al., 2001). In contrary, data from VPAC2 knockout mice manifested a marked reduction in IL-4, IgG, IgE and tendency to delayed-type hypersensitivity (Goetzl et al., 2001).

These studies may therefore suggest that activation of VPAC2 may initiate or direct the immune system towards a Th2/allergic response.

1.3.3.3 PAC1

PAC1 is prevalent in CNS tissues (Joo et al., 2004). It is also found in many peripheral organs such as adrenal medulla (Reubi, 2000), rat thyroid and parathyroid glands (Fahrenkrug, 2010). In immune cells PAC1 receptor is constitutively expresses in human monocytic THP1 cells (Delgado and Ganea, 2001b, Delgado et al., 2003) and murine peritoneal macrophages (Pozo et al., 1997, Delgado et al., 1998).

PAC1, along with VPAC2, induces vasodilation in human skin following heat stress (Kellogg et al., 2010). Martinez et al. (2002) investigated the anti-inflammatory role of PAC1 in murine model of endotoxic shock

by using PAC1 -/- KO mice. The study suggested that PAC1 expression was crucial in inhibiting overproduction of IL-6 and nitric oxide (NO) production in mouse serum following administration of LPS. Later, the group (2005) reported that VIP or PACAP (via PAC1 ligation) also inhibits neutrophil activity and expression of adhesion molecules when these mice were exposed to a lethal dose of *Salmonella* LPS. Furthermore, the study also stated that PAC1 had a crucial role in myeloperoxidase reduction in intestine and liver extracts of LPS-administrated mice. Findings also revealed PAC1 inhibitory effect on wild type mice expression of adhesion molecules on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM), compared to PAC1 -/- KO mice (Martínez et al., 2005).

1.3.4 Down-stream signalling pathways activated by VPAC receptors

As stated earlier, VIP receptors belong to the class II GPCRs, which translocate their signals via G-protein stimulation. Coupling of VIP or PACAP with VPAC receptors induces phosphorylation of GDP to GTP, on the G protein, which then initiates down-stream signalling pathways via cAMP-dependent and independent pathways. VPAC mediate their effects through the three subunits (α, β and γ) of the G protein, proteins, which are, involved in transducing extracellular signals into down-stream biochemical cascades. Gα subunits also consist of three sub proteins (Gαs, Gαq or Gαi). It is via the Gαs subunit VPAC mediates the most important signalling pathway through induction of the adenylate cyclase, cyclic adenosine monophosphate (cAMP) and PKA pathways (Dickson and Finlayson, 2009) which then mediate most of the anti-inflammatory actions attributed to VIP and PACAP (Delgado et al., 1999d). Most cAMP-elevating factors have been shown as powerful anti-inflammatory agents by reducing Th1 reaction, generally through down-regulating NF-κB pathway, and activating regulatory T cells (Gonzalez-Rey et al., 2007a). Some studies have focused on the effect of VIP on the MAPK pathway, as a down-stream signalling pathway activated by the cAMP-PKA pathway, in human immune cells (Harfi et al., 2005, El Zein et al., 2006). These studies have shown a stimulatory effect of VIP on the MAPK pathway, particularly via VPAC1. These studies suggested that VIP activates ERK

and P38 MAPK in human monocytes and this was confirmed by using their inhibitors PD98059 and SB203580, respectively. VIP-induced MAPK pathway enhances proliferation and secretion of pituitary cells (Le Pechon-Vallee et al., 2000) as well as proliferation and differentiation of neuroendocrine cells (Gutierrez-Canas et al., 2006, Emery and Eiden, 2012). The second, essential, pathway activated by VIP is the phospholipase (PLC) pathway, which subsequently elevates intracellular calcium concentration initiated by the interaction of VPAC to Gαq or Gαi. It is been shown that all VIP receptors (VPAC1, VPAC2 and PAC1) are able to elevate intracellular calcium levels endogenously (Dickson et al., 2006, Langer, 2012) as well as in transfected cells (Sreedharan et al., 1994). Increased cystolic calcium concentration are attained by the ability of VIP/VPAC to mobilise Ca^{2+} either from inositol trisphosphate stores or regulation of calcium channels on the cell membrane (Vaudry et al., 2000). Several studies have examined the elevated calcium level in VIP-treated cells and have shown that VIP stimulates exocytosis and secretion of many hormones and neurotransmitters. For instance, insulin from pancreatic beta cells (Sanlioglu et al., 2012), catecholamine (Henning and Sawmiller, 2001), growth hormone from the pituitary gland (Vijayan et al., 1979) as well as stimulating secretory granules in the trachea, duodenum and testis (reviewed in El Zein et al., 2008). The schematic diagram of VIP/VPAC signalling transduction is shown in figure 1.5.

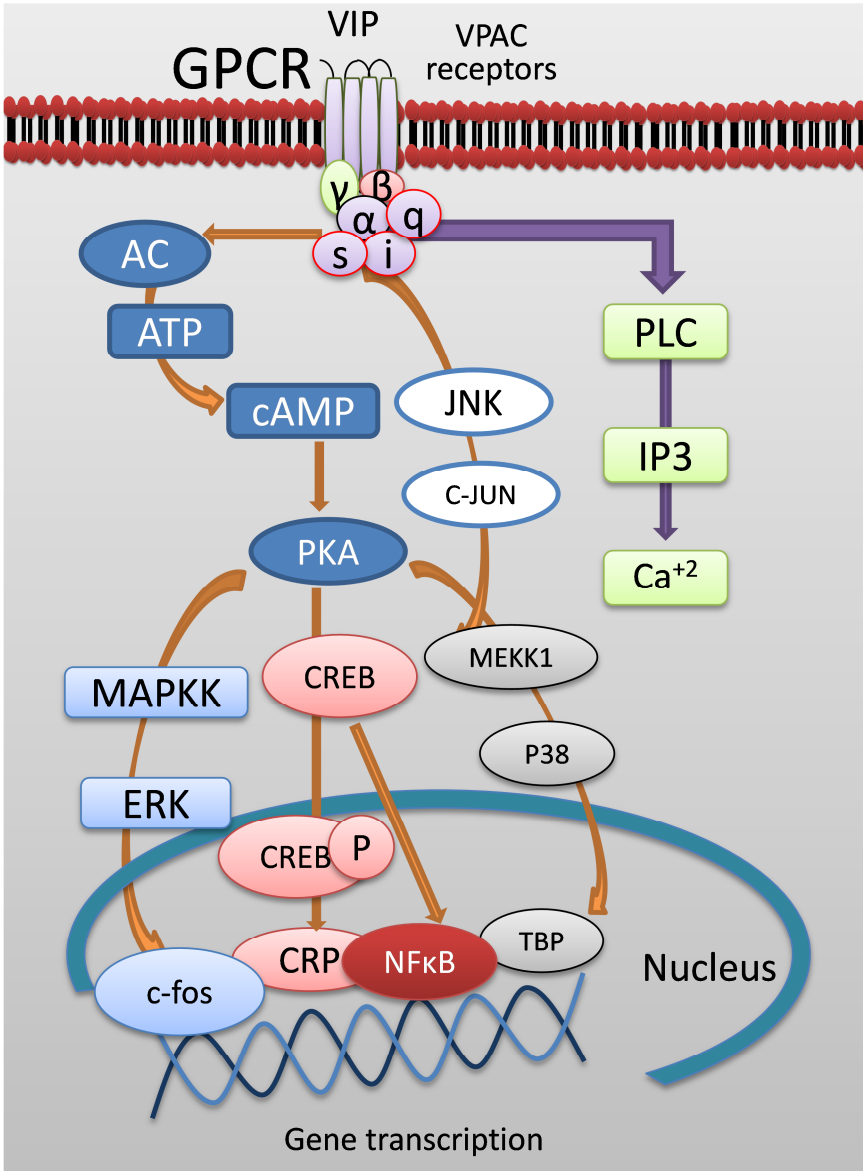

Figure 1.5: VPAC receptor signalling pathway.

VIP/VPAC binding (Gαs) induces adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). PKA activate some inflammatory transcription factors such as NF-κB and c-for. Also, VIP stimulate cAMP response element binding protein (CREB) through Jun N-terminal kinases (JNK) and Extracellular signal-regulated kinases (ERK). Moreover, VPAC through Gαq or Gαi trigger of phospholipase (PLC) and subsequently and increase intracellular calcium concentration.

1.3.5 VPAC internalisation mechanism

Upon stimulation, VPAC1 and VPAC2 are promptly phosphorylated, which initiates a signalling cascade, prior to a desensitisation process which controls extra signalling (McDonald et al., 1998). In intestinal smooth muscle (Murthy et al., 2008) and African monkey kidney (COS7) (McDonald et al., 1998)cells, it has been shown that VPAC2 receptors are phosphorylated via GPCR kinase (GRK) and PKA. While the desensitisation process occurs via detachment of the VPAC2 from G proteins, most likely due to the effect of arrestin (McCulloch et al., 2002). Arrestin, is a small regulatory protein which blocks G Protein-induced signal cascades and then induces internalisation of the receptor via clathrin-coated vesicles (Hopkins et al., 1985). Clathrin surfaced pits or endosome are formed by concentrating of GPCR ligand-receptor complex (von Zastrow, 2003). The early endosome can receive cargos from both the internalised or transported vesicles of the Golgi apparatus then organise it in budding vesicles (Barysch et al., 2009). The formation of endosomes is governed by GTPases, particularly Rab5a which is crucial for soluble *N*-ethylmaleimide-sensitive factor (NFS) attachment protein receptor (SNARE) formation, which is an enormous protein superfamily that regulates vesicle trafficking and fusion (Hutagalung and Novick, 2011). Moreover, Rab5a recruits early endosomal antigen1 (EEA1), a tethering protein that docks and fuses vesicles to SNARE, subsequently leading to endosomal shipment (Chia and Gleeson, 2014). Localisation of EEA1 on the early endosome is regulated by a C-terminal domain which can bind to

Rab5a, calmodulin and inositol 3-phosphate (Dumas et al., 2001). Rab5a is the most essential protein which regulates and matures the early endocytic vesicles prior to the formation of the late endosome (Rink et al., 2005). Generally, the internalised GPCR undergoes two endocytic routes. It either directs the internalised materials into late endosomes, which mature and localise in the cell cytoplasm, prior to transportation to the perinuclear region where they fuse with lysosomes (Maxfield and McGraw, 2004). The late endosomes are coated with lysosomal associated-membrane proteins (such as LAMP1 and LAMP2) and acidic hydrolases which degrade the majority of endosomal components, which subsequently ends the receptor signalling (Luzio et al., 2000). Alternatively, the receptor may be recycled instantly to the cell membrane, by Rab4 or Rab5, and bind again to G proteins, or it is recycled slowly via a recycling endosome and Rab11 (Cheng and Filardo, 2012). Rab11, regulates two-way material exchanges between the early endosomes and the trans-Golgi network, the major sorting site of protein (Wilcke et al., 2000, Huotari and Helenius, 2011). Rab11 (subunits a, b, and c) mainly localise on the recycling endosome and also on the trans-Golgi network (TGN) compartment surfaces (Chen et al., 1998, Sönnichsen et al., 2000). Immature secretory granules (containing protein) bud from the TGN are transported to the PC12 cell (a cell line developed from the adrenal medulla of rats) margins where they mature (over 16 hours old) and are then exocytosed (Rudolf et al., 2001). By using total internal reflection fluorescence microscope (TIRFM) in PC12 cells, it was shown

that Rab27A and Rab3a are associated with newly formed secretory granules (Duncan et al., 2003) and Rab3a remains associated throughout the different developmental stages (Handley et al., 2007).

1.3.6 Clinical Significance of VIP

As pleiotropic peptide, VIP role to regulate immune response during chronic and autoimmune disorder has been suggested by many authors. Main VIP effects are through balancing host immune response by decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines in response to infection (POZO and DELGADO, 2004, Gonzalez-Rey et al., 2007a). In addition, VIP has protective effects which, reduce cell death in CNS tissues (Morell et al., 2012) and also mediate vasodilation and bronchodilation (Mathioudakis et al., 2013).

The VIP has been shown to have clinical potential in the treatment of many different inflammatory diseases:

1.3.6.1 The therapeutic potential of VIP in septic shock

To date, most studies investigating the effect of VIP on the production of pro-inflammatory mediators have either used mouse or human macrophages or monocytes cultured with LPS *in vitro*, or *in vivo* administration of LPS, followed by VIP, in a murine model of endotoxaemia.

1.3.6.1.1 VIP modulates the host immune response by decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines in response to LPS

In an LPS-induced, murine model of sepsis, VIP has been shown to suppress cytokine and chemokine production. For example, it has been shown that in an LPS-induced sepsis model in BALB/c mice,

intraperitoneal administration of VIP or PACAP (5 nmol/mice) reduced serum and peritoneal fluid TNF-α and IL-6 concentrations by around 50% (Delgado et al., 1999a). In this latter study, it was also shown that administration of VIP was also associated with amelioration of histo-pathological changes in kidney, spleen, lung and an increased survival rate of 60%. The suppression of LPS-induced cytokines by VIP in mice occurred via VPAC1 ligation and down-stream inhibition of NF-κB, c-Jun, CREB and cAMP pathways (Delgado et al., 1998). Furthermore, VIP and PACAP have been shown to reduce IL-6 production from LPS cultured murine peritoneal macrophages (Martinez et al., 1998).

The formation and elimination of reactive oxygen species (ROS) is balanced in normal physiological status. Oxidative stress occurs when the production of oxidant compounds exceed their removal (Nathan and Singer, 1999). This condition may accompany sepsis and cause oxidation of protein, change in membrane permeability and ultimately may cause organ damage (Macdonald et al., 2003). VIP has been demonstrated to reduce NO secretion after LPS stimulation of cells *in vitro* and *in vivo*. For example, VIP and PACAP decreased NO production from peritoneal macrophages stimulated with LPS or IFN-γ and, in this study, the inhibitory role of VIP correlated with both decreased IFN-γ regulatory factor 1 and NF-κB transduction pathways (Delgado et al., 1999c). Foster et al. (2005b) investigated the role of VIP on virulent and avirulent *S*. Typhimurium survival in murine macrophages in the presence or absence of IFN-γ. The study shows

that VIP (10^{-10} M) suppressed gp91^{phox} expression, a primary trans-membrane protein from cytochrome b558 subunits which translocate with other cytosolic components such as p40^{phox}, p47^{phox} and p67^{phox} to induce ROS pathways. VIP also supressed the production of ROS but this was associated with significantly increase intracellular bacterial growth, even in the presence of IFN-γ, which usually enhances macrophage killing pathways and *Salmonella* death (Foster et al., 2003).

In addition to the suppressive role of VIP and PACAP on the production of pro-inflammatory cytokines induced by LPS, it was also reported that both peptides raised peritoneal fluid and serum IL-10 production via the CREB pathway in murine macrophages challenged with LPS in both *in vitro* and *in vivo* (Delgado et al., 1999b). The increase in IL-10 production by VIP has also been shown to induce the development of tolerogenic DCs and the subsequent proliferation of T suppressor cells following DC/T cell co-culture (Delgado et al., 2005). In rats, administration of VIP (5 nmol) was also shown to increase serum IL-10 and reduce TNF-α and IL-1β levels, following administration of LPS (Zhang et al., 2006). In this model the severity of tissue pathology (measured by necrosis, haemorrhage and leucocytosis) was also reduced by VIP in rat intestines (Zhang et al., 2006) and lungs (Zhang et al., 2010).

1.3.6.1.2 VIP regulates the TLR activity induced by LPS

VIP has been shown to reduce TLR expression in immune cells stimulated with LPS in various host species. A study by Zuo et al. (2010)reported that VIP inhibited up-regulation of both TLR2 and TLR4 mRNA expressions in rat alveolar macrophages stimulated by *E*. Coli LPS. In support of this, Arranz et al. (2008) showed the importance of VIP in a trinitrobenzene sulfonic acid (TNBS) -induced murine model of colitis. In this study, VIP returned TLR2 and TLR4 expressions to normal states in $CD4^{+}CD25^{+}$ T lymphocytes, by enhancing Foxp3 protein which orchestrates Treg functions and inhibited production of IL-12 and IFN-γ.

Therefore, it has been shown that VIP possesses therapeutic potential in Gram-negative endotoxaemia and sepsis in both murine and human models of disease. Studies have indicated that VIP inhibits the production of pro-inflammatory mediators by macrophages and monocytes at many different levels. However, very little has been published regarding the effect of VIP on these mediators in human monocytes cultured with viable Gram-negative bacteria, or in murine models in which the animals are infected with virulent bacteria rather than injected with LPS.

1.3.6.2 Rheumatoid arthritis (RA)

To date, many studies have affirmed the role of VIP in the therapeutic potential of chronic inflammatory and autoimmune diseases. Foey et

al. (2003) investigated the effect of VIP on the production of inflammatory mediators from monocytes and macrophages isolated from the synovial tissues of RA patients. The study reported that VIP (dose dependently) decreased TNF-α and IFN-γ production and inhibited T cell activity. In murine, VIP exerted inhibitory effects, confirmed by both ELISA and PCR, on IL-8 and monocyte chemotactic protein 1 (MCP-1)(Juarranz et al., 2004), IL-1β, and TNF-α (Juarranz et al., 2005). In human rheumatic synovial cells, VIP down-regulated mRNA expression of IL-6 cells (Juarranz et al., 2008) and expression of TLR2 and 4 and on MYD88 when the cells stimulated by LPS (Gutierrez-Canas et al., 2006).

1.3.6.3 Asthma

The anti-oxidant role of VIP was examined in VIP knockout mice. The anti-oxidant protein expression level in lung, carbonyl reductase, was measured by genome microarray and it was highly expressed in the VIP-deactivated mice. The study concluded that absence of VIP in mice resulted in increased pulmonary inflammation and rendered mice more susceptible to asthma (Szema et al., 2011). Morice et al. (1984) explored the role of VIP in 7 patients with asthma. The study shows that VIP reduced histamine concentration and induced bronchodilation following 15 minutes from VIP administration. A study by Wu et al. (2011) suggested that VIP ameliorated lung disease though increased

airway and pulmonary vessel relaxation and enhanced circulation in the pulmonary tissues.

Owing to its short half-life, dilatation of guinea pig airways, by VIP only lasted about 10 minutes (Lindén et al., 1998). Therefore, a polymer grafted liposome was used to deliver the peptide into lung tissues and save VIP from proteolytic degradation (Stark et al., 2007). In asthmatic patients, inhalation of 600 µg of the synthetic VPAC2 agonists (Ro 25-1553) resulted in immediate bronchodilation and this is likely a promising candidate treatment for asthmatic people (Linden et al., 2003).

1.3.6.4 Crohn's disease

The therapeutic potential of VIP in the colitis has also been studies. In murine models of colitis, induced by oral administration of TNBS, VIP ameliorated both the histopathological and clinical signs of colitis (Abad et al., 2003, Gonzalez–Rey and Delgado, 2006). The effect of VIP in this model (to maintain immunological homeostasis) likely occurs via inhibition of NF-κB (Tao et al., 2007, Shi and Sarna, 2009) and/or TLR2 and TLR4 (Gomariz et al., 2005, Arranz et al., 2006) which inhibits down-stream hyper-production of IFN-γ, TNF-α, and IL-6 (Abad et al., 2003). The effect of VIP on intestinal, immunological, homeostasis has also been studied using VIP KO mice. VIP KO mice have distorted intestinal shape and size, including reduced weight and thickness of smooth muscle and also reduction in goblet cell number

(Lelievre et al., 2007). Due to the protective role of mucous, secreted by goblet cells, this latter finding probably has a direct effect on inflammation by reducing the protective barrier during the hyper-inflammatory state. When VIP KO mice were inoculated with dextran sodium sulphate, a model of human inflammatory bowel disease, increased expressions of several cytokines and chemokines, such as IL-4, IL-6 and IL-17 (which regulates T cell migration), were measured in the VIP-knockout mice compared with wild type control mice (Yadav et al., 2011).

1.4 Aims of the study:

The overall aims of the research described in this thesis were:

1. To investigate the effect of the VIP on the growth dynamics of *S*. Typhimurium in human monocytes and the effect of VIP on production of inflammatory mediators by these cells.

2. To examine the localisation and expression of VPAC receptors (VPAC1, VPAC2 and PAC1) in human monocytes challenged with either *S*. Typhimurium or LPS in the presence or absence of VIP.

3. To determine the impact of the *Salmonella*, LPS, or VIP on the intracellular transport and cycling of the VPAC1 receptor in human monocytes.

Chapter Two

2. Methodology

2.1 Human monocytes

2.1.1 Media and growth condition

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) strain 4/74 (Jones et al., 1988, Paulin et al., 2007) was used for all experiments.

Nutrient agar and nutrient broth (Oxoid, UK) were prepared and used for growth and determination of the bacterial numbers. A streak from a *S*. Typhimurium nutrient agar slope was inoculated into a 10 ml volume of nutrient broth in a universal bottle and incubated statically at 37°C for 24h in accordance to a study by Barrow and Lovell (1989). Then, 3 loops from the incubated nutrient broth were sub-cultured into nutrient broth warmed to 37°C and placed in a shaking incubator (Forma orbital shaker-Thermo, UK) at 37°C shaking at 150 rpm for 18h. After 18h, 100 µl was withdrawn and put into a fresh pre-warmed 10 ml volume of nutrient broth, which was then incubated for a further 4h in a shaking incubator under the conditions above.

A growth curve was determined by enumerating colony-forming unit (CFU) from serial dilutions and optical density measurements at 600 nm wavelength in a spectrophotometer (Scientific laboratories supplies, Hessle, UK) at 2-hour intervals during incubation. Following bacterial growth curve and also optical density data, 1 ml of bacterial

suspension at 4h was diluted with phosphate buffer saline (PBS) to give an MOI of 10:1 to human monocyte (Kaiser et al., 2000).

2.1.2 Isolation of blood monocytes

Human blood (50 ml) was purchased from the blood transfusion service (Sheffield, UK). The blood, PBS and Histopaque-1077 (Sigma, Poole UK) were pre-warmed to room temperature. The blood was diluted with the same amount of sterile PBS (1:1 v/v) then gently poured onto Histopaque-1077, 1 volume of mixed blood/PBS:1 Histopaque. Later, the suspension was centrifuged at 400xg at ambient temperature for 30 mins using an Avani-JA centrifuge (Beckman Coulter, UK). The buffy coat layer was then removed by Pasteur pipette, re-suspended with PBS and centrifuged again at 250g for 10 mins. Subsequently, RBC lysis buffer ($KHCO_3$ 1 M, NH_4CL 0.15 M and EDTA 0.1 M) was added (10:1 v/v) to the suspension, vortexed and left for 15 mins prior to centrifugation; when necessary this step was repeated. Supernatants were discarded and re-suspended with 80µl of cold MACS buffer (2 mM EDTA and 0.5 % BSA were added to PBS then their pH adjusted to 7.2) and 20 µl of anti-CD14 antibody coated micromagnetic beads (Miltenyi Biotech, Auburn, USA) for every 1 x 10^7 monocytes. Afterwards, the cell suspension was left 15 minutes on the orbital shaker, in the dark, at 4° C. The suspension filled 10 ml of cold MACS and centrifuged at 250g for 10 minutes. The supernatant was discarded again and the pellet re-suspended in 5 ml

of the cold MACS buffer. Then, a separation column (LS; Miltenyi Biotech, UK) was inserted into a magnetic separator and 3 ml of the cold MACS buffer rinsed through the column. Next, the cell suspension passed the column followed by washing the column three times with 3 ml of the cold MACS buffer. Five ml of MACS buffer were placed onto the column and push the column plunger to load positive selection cells.

To determine monocyte purity, 10 µl of FITC-conjugated mouse anti human CD14 antibody (AbD serotec, KidlingtonOxon, UK) were incubated with the isolated cells and left on the orbital shaker in the cold room (4° C) for 30 minutes. Then, the suspension directed to FACSCanto™ II system (BD Bioscience, USA) and the FACS analysis detected over 90% cells expressing CD14 positive. 50 ml of complete RPMI media (Gibco, Paisley, UK) containing 5ml of foetal bovine serum (10% v/v), 0.5 ml of L- glutamine (2mM), 0.5 ml of streptomycin (100 µg/ml) and penicillin (100 U/ml) (Sigma-Aldrich, USA) were added to the pellets. Cell viability and numbers were then assessed staining with trypan blue and using a haemocytometer. Cell viability was found to be more than 90%. Cells were then aliquoted into 24 well plates and incubated at 37°C in 5% CO_2 to allow for monocyte adherence overnight, prior to the bacterial invasion assay.

2.1.3 Bacterial invasion assays

The cultured monocytes were first washed with sterile PBS to eliminate the effect of complete RPMI media then the bacteria were added to the cultured monocytes at a MOI of 10:1, based on protocol reported in previous studies (Chen et al., 1996, Brennan and Cookson, 2000, Smalley et al., 2009, Forest et al., 2010, Strandberg et al., 2012), then cultured at 37°C, (5% CO_2) for 60 mins. The culture was washed and 1 ml RPMI media containing 100 µg/ml of gentamicin (Sigma-Aldrich) with or without VIP at concentration of 10^{-7}M (V6130; Sigma-Aldrich) was then added into the wells and placed in the incubator for a further 60 mins. The cultured cells were washed again and the medium was substituted with RPMI containing 25 µg/ml of gentamicin with or without VIP (10^{-7} M) for 2, 6 and 24h post-culture. At each time point (2, 6 and 24h) the cell supernatants were gently aspirated (for measuring cytokines). Subsequently, the cell monolayers were lysed by the addition of 200 µl of Triton X (1% v/v) for a further 15 minutes. Finally, 20 µl of lysed culture was seeded into 180 µl of PBS in 96 well plates to perform serial dilutions to ascertain growth at the different time points used in the experiments.

2.1.4 Stimulation of human monocytes with LPS and VIP

LPS of *S*. Typhimurium SL1181 at concentration of 100 ng/mL (Sigma-Aldrich, Dorset, UK) was used to stimulate 5 x 10^5 cells/mL in 24-well plates for 2, 6 and 24 h post exposure. The concentration of the LPS

was chosen as optimal inducer for cytokine production (Shimauchi et al., 1999, Sinistro et al., 2007, Ibrahim et al., 2011, Ngkelo et al., 2012, Kubo et al., 2013). In parallel experiments, VIP (10^{-7} M) was added to LPS-treated monocytes at the same time. Then supernatants of the cultured monocytes at different time points were removed and stored (-20 ^{0}C) until used.

2.1.5 Monocyte survival assay

Monocytes were also tested for viability with propidium iodide (PI) in accordance with Sasaki et al. (1987). Freshly isolated monocytes (1 x 10^{6}) were seeded into well plate then the invasion assay carried out with either *S*. Typhimurium (at MOI 10:1) or LPS (100 ng/mL) in the presence or absence of VIP (10^{-7} M) for 24h. Cold absolute methanol (Fisher Scientific, UK) was added to the cells for 30 mins after removal of the medium and used as positive control. After monocyte detaching from the cultured well-plate with Accutase cell dissociation agent (Life technologies, UK), the cells were centrifuged at 300g for 8 mins. Afterwards, 1ml of cold PBS containing 10 µg/ml of propidium iodide (Molecular probes, Life technologies) was added, mixed well and then held in the dark at 5 °C for 10 mins. The cell population were gated out to appropriate forward scatter (FSC) versus side scatter (SSC), and then samples were acquired by CellQuest pro software. The fluorescence data analysis was performed by FACS Diva software (BD Bioscience, USA).

2.1.6 Measurement of cytokines using enzyme-linked immunosorbent assay (ELISA)

The supernatants of cultured-human monocytes, which had been treated with *S*. Typhimurium (4/74) strain or LPS (with or without VIP) were stored in -20 ^{0}c until used. The concentrations of IL-1β, IL-6, TNF-α (DuoSet, R&D Systems, Oxford, UK), IL-4 and IL-10 (R&D Systems, Inc., USA) in the cell supernatants were measured by using commercially available ELISA kits (R&D Systems, Oxford, UK) as per the manufacturer's instructions. Briefly, 100 µl of capture antibody was coated in 96 well plate sealed and then incubated overnight at room temperature. The plate was washed 3 times with washing buffer (Tween® 20 added to PBS). The plate was then blocked by adding 300 µl of reagent diluent (R&D Systems, UK) for an hour and then washed again. One hundred µl of sample was loaded into the well-plate and left for 2h. After washing steps, detection antibody (100 µl) was added to each well, sealed and incubated for 2h. Afterwards, 100 µl of horse-radish peroxidase enzyme (HRP)-streptavidin conjugate was added and the plate left in the dark for 20 mins. Then, 50 µl of substrate solution (H_2O_2 and tetramethylbenzidine mixture) for 20 minutes away from direct light. This was followed by adding 50 µl of stop solution (2M H_2SO_4). Finally, optical density was measured directly with a microplate reader (LT-4000, Labtech, UK) and wave lengths were set according to manufacturer's instructions. All experiments were performed in triplicate on 3 separate occasions.

2.1.7 Immunofluorescence

Glass cover slips (14 mm size, VWR international LTD, UK) were firstly cleaned by placing in 1M HCl in an oven 50 °C overnight. The coverslips were then rinsed three times with distilled water and sterilised by autoclaving. Afterwards, 5 x 10^5 of freshly-isolated monocytes were seeded onto the cover slips and incubated for a day at 37°C, (5% CO_2) before carrying out the invasion assay. The cells were gently washed with PBS and then fixed by 4% paraformaldehyde at room temperature for 15 mins. To permeabilise the cells, they were again washed and incubated with 0.2 Triton X-100 in PBS for 10 mins on ice box. After washing, they were blocked with 3% bovine serum albumin (Fisher Scientific, Loughborough, UK) in PBS at room temperature for 30 mins and washed again. After washing steps (3 times) with the washing buffer, 200 µl of diluted phalloidin–tetramethylrhodamine B isothiocyanate (Sigma-Aldrich, Dorset, UK) in PBS was added to the cells and left in the dark for 20 mins. Following washing with PBS, the coverslips were mounted onto a clean slide with Vectashield Hard-Set Mounting Medium labelled with DAPI (Vector laboratories, Inc., Burlingame, USA). The visualisation and expression of receptors were done with a Leica DM 5000B microscopy (Leica microsystems ltd, Germany). Different microscope fluorescence filters, which were excited at different wavelengths, were used: blue for DAPI, green for FITC and red for phalloidin.

2.1.8 Co-localisation of VPAC1

Freshly isolated monocytes (5 x 10^5) were seeded on sterilised cover slips and left for a day before an invasion assay carried out with *S*. Typhimurium (at MOI 10:1) or LPS (100 ng/mL) in the presence or absence of VIP (10^{-7} M) for 24h. The cells/cover slips were gently rinsed with PBS and subsequently fixed by 4% paraformaldehyde at room temperature for 15 mins. The cells were then washed three times and incubated with 0.2 Triton X-100 in PBS for 10 minutes, washed again (3 times) and then blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 30 mins followed by washing (2 times). Cells were then incubated for 60 mins with the appropriate primary antibody followed by the addition of secondary antibodies as shown in Table 2.1. The cover slips were left for 45 mins in the dark, and then washed extensively (3 times) with washing buffer (Tween® 20 in PBS). Finally, Vectashield Hard-Set Mounting Medium labelled with DAPI (Vector laboratories, USA) mounted coverslips onto clean slides. The visualisation and expression proteins were done with a confocal scanner laser SP2 (Leica Microsystems, Heidelberg GmbH). Immunofluorescence images were acquired using a correct wavelength setting. Images were taken at 4µm bottom to the monocytes surfaces.

2.1.9 Flow cytometry (FACS)

Purified monocytes (5 x 10^5) were washed twice with chilled PBS and detached from the cultured well-plate with Accutase cell dissociation agent (Life technologies, UK). The cells were centrifuged 300xg for 10 mins and supernatants were discarded. One ml of 4% paraformaldehyde was added to the permeabilised cell suspension, mixed well and left on ice for 10 mins. The suspension was then rinsed with chilled PBS, centrifuged and the pellet re-suspended in 1ml of 0.2% Triton x-100 (Fisher Scientific, Loughborough, UK) on ice for 10 mins. After centrifuging and further washing steps (3 times), all cell suspensions were re-suspended in cold FACS buffer (3% BSA+PBS) and held for 30 mins at room temperature to block non-specific binding. Subsequently, the cells were centrifuged, and then 300 µl of the primary antibody was added, mixed well and placed onto am orbital shaker for 60 mins. Next, the cells were centrifuged and washed 3 times with cold PBS, incubated with 300 µl of appropriate secondary antibody (as per Table 2.1) in the dark, on an orbital shaker for 45 minus. The cells were centrifuged and washed with washing cold PBS then analysed with the FACScan™ flow cytometer system (Becton Dickinson, Bioscience, USA). The cell population was gated out to the appropriate forward scatter (FSC), side scatter (SSC), setting. Finally, the fluorescence data analysis was performed by FACSDiva software (BD Biosceince, USA) and the quadrant setting was based on isotype control autofluorescence.

Receptor	Primary Antibodies	Conc. µg/ml	Secondary Antibodies (Conjugated)	Conc.µg/ml
VPAC1	Mouse monoclonal anti human VPAC1-IgG2a (Abcam, Inc., Cambridge, UK)	1 µg	Rat monoclonal to mouse IgGa2-FITC (Abcam, Inc., Cambridge, UK)	0.1 µg
VPAC2	Rabbit polyclonal to human VPAC2 (Abcam,Cambridge Inc., UK)	5 µg	Goat anti Rabbit - FITC (Abcam, Inc., Cambridge, UK)	2 µg
PAC1	Mouse monoclonal anti human PACAP-IgG1(Abcam, Inc., Cambridge, UK)	5 µg	Mouse IgG1-FITC conjugated(Invitrogen Frederick, USA)	1 µg
LAMP1	Rabbit polyclonal anti human LAMP1-IgG(Abcam, Inc., Cambridge, UK)	5 µg	Donkey anti rabbit IgG-Alexa four (647) conjugated. (Abcam, Inc., Cambridge, UK)	2 µg
EEA1	Rabbit anti-human EEA1-IgG (Abcam, Inc., Cambridge, UK)	1 µg	Donkey anti rabbit IgG-Alexa four (647) conjugated. (Abcam, Inc., Cambridge, UK)	2 µg
Rab3a	Rabbit anti-human Rab3a-IgG (Abcam, Inc., Cambridge, UK)	1 µg	Donkey anti rabbit IgG-Alexa four (647) conjugated. (Abcam, Inc., Cambridge, UK)	2 µg
CaM1	Mouse anti-human CaM1 – IgG1(Abcam, Inc., Cambridge, UK)	2 µg	Mouse IgG1-FITC conjugated(Invitrogen Frederick, USA)	1 µg
Rab11a	Rabbit polyclonal to human Rab11a (Abcam, Cambridge Inc., UK)	1 µg	Donkey anti rabbit IgG-Alexa four (647) conjugated. (Abcam, Inc., Cambridge, UK)	2 µg
TGN46	Mouse monoclonal anti human TGN46-IgG1(Abcam, Inc., Cambridge, UK)	50 µg	Mouse IgG1-FITC conjugated(Invitrogen Frederick, USA).	1 µg

Table2.1: List of primary and secondary antibodies for the immunofluorescence and flow cytometry work (2.1.9).

2.1.10 RNA Extraction

Total RNA was purified from cultured human monocytes (3 x 10^6 per well) using the RNeasy plus kit (Qiagen, Hilden, Germany). The cells were first washed by PBS (3 times) and detached from the cultured plated using a cell scrapper (Orange Scientific, Belgium). The suspensions were centrifuged at 10000g for 5 mins, the supernatant discarded and the pellet re-suspended in 350 µl of RLT buffer (before use 10µl of β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) was added to each 1 ml of RLT Buffer) and vortexed for 30 seconds (s). Afterwards, the cell lysates were transferred to QIAshredder spin columns (provided with the kit) and centrifuged at 13000g for 2 min. The column tops were thrown away and 350 µl of 70% ethanol was added to the suspension and mixed thoroughly. To remove any DNA contamination, the suspensions (700 µl) were transferred into genomic DNA (g DNA) columns and centrifuged for one minute. The suspensions were then transferred to a RNeasy spin column and centrifuged at 10000g for 30 s and the flow-through discarded. Then, 700 µl of RW1 buffer (supplied with the kit) was added in the column, centrifuged and the flow-through again discarded and this step was carried out twice. To rinse the column membrane, 500 µl of RPE buffer (supplied with the kit) was added to the column, centrifuged at 10000g for 30 s and the flow-through discarded. The last step was repeated with the same buffer concentration, but with a 2 min centrifugation time and the flow-through discarded. Finally, the column tops were

transferred to collection tubes and 30 µl of RNase free water was added to elute RNA.

2.1.11 Complementary DNA (cDNA) synthesis

Although Agilent bio-analyser is being largely used for detection of RNA integrity, which is used greatly for transcriptional, sequencing of DNA and RNA library (Fleige and Pfaffl, 2006, Romero et al., 2014), some studies states that 260/280 ratio in NanoDrop analysis is acceptable if it is higher than 1.8 (Manchester, 1996, Clark and Christopher, 2000).

The amount and quality of total extracted RNA samples were measured by NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, UK). Good quality RNA samples were determined via 260/280 ratio, which ranged from 1.8 - 2.2). Then, 100ng RNA specimens were converted into complementary DNA according to the SuperScript™ First-Strand Synthesis kit's instructions (Invitrogen, Carlsbad, CA, USA). In the first reaction, RNA (100ng), 1 µl of 10 mM dNTP mix, 2 µl random hexamers at concentration of 50 ng/µl and DEPC-treated water (added 10 µl final volume) were placed into a sterilised Eppendorf tube. These components were mixed thoroughly, placed on a heat block at 65 °C for 5 mins and were then chilled on ice. In the second reaction, 2 µl 10 x RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT and 1 µl RNase OUT™ (Invitrogen, Carlsbad, CA, USA) at concentration of 40 U/µl were used for each sample. These components were mixed well and centrifuged

briefly. Then, 9 µl of the second reaction were placed in each Eppendorf tube of the first reaction and then incubated for 2 mins at room temperature. After that, 1 µl of SuperScript™ II RT (supplied with the kit) was added to each Eppendorf at room temperature for 10 mins. Then, the reactions were placed on a heat block at 42°C for 50 mins, the 70°C for 15 mins then finally chilled on ice. Finally, 1 µl of RNase H (supplied with the kit) was added to each reaction, which was heated again at 37°C for 20 mins and immediately stored at -20°C.

2.1.12 Agarose gel electrophoresis

The purified RNA samples were reverse-transcribed by Superscript III one-step qPCR with platinum Taq (Invitrogen, Life technologies, USA) according to the manufacturer's guidelines. The reaction consisted of 25 µl of the 2X Reaction Mix, 50 ng of RNA, 1 µl of 10uM forward primer, 1 µl of 10uM reverse primer and 1 µl of Platinum Taq polymerase (Invitrogen, Life technologies, USA). These components were mixed and sterilised distilled water was added to 50 µl final volume. Samples were placed into XP thermal cycle (Bioer Technology; Alpha laboratories, UK) for the RNA amplification process. The amplification program was set according to the manufacturer's instructions. First, the DNA template was denaturated by holding at 94 °C for 2 mins. After that, a total 40 cycles of 94 °C for 15 s, 60 °C for 30 s and then amplification for 68 °C for 1 min. Next, the reaction was held for 10 mins at 68 °C and finally cooled for 5 mins at 4 °C.

To make 1% gel agarose gels, 1g of agarose (Fisher Scientific, Loughborough, UK) was added to 99 ml of TAE buffer (0.001 mM EDTA and 0.04 mM Tris acetate at pH 8). The mixture was dissolved in the microwave and then 5 µl of 0.5 mg/ml ethidium bromide (Sigma-Aldrich, USA) was added to it. An appropriate comb was inserted into the casting tray and the mixture was poured gently into it. Then the gel was left to dry, the comb gently removed and the casting tray was then submerged in TAE buffer. Meanwhile, 3 µl of loading buffer (0.25% xylene cyanol and 0.25% bromophenol blue) was added to 12 µl of each sample, mixed well and carefully loaded into the wells of the casting tray (Thermo Scientific, Portsmouth, USA). The gel was run at 100 volts for 45 mins. Finally, the DNA samples were visualised by UV light, and photography performed using an Image Quant 300 imager (Amersham Biosciences, UK).

2.1.13 Real time PCR

The PCR reaction volume was 20 µl, consisting of 10 µl of Light Cycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 1 µM (300 nM) of each forward and reverse primer (Eurofins MWG Operon, Germany), 0.2 µl of universal probe library (Roche Applied Science), labelled with fluorescein and dark quencher dye, and the remaining was made up by PCR water. Primers and probes were designed using universal probe library (Roche Diagnostics) as shown in table 2.2. In control negative wells, PCR water was added to the other

reagents instead of cDNA. The standard curves of the target and reference genes were consisted of five serial 10-fold dilutions from 1:10 to 1:100000. The reagent mixtures were loaded into 96 well-plates (Roche Applied Science). The thermal cycles consisted of initial denaturation at 95°C for 10 mins and 40 cycles of sample amplification. This started with 95°C for 10 s, 60°C for 30 s, 72°C for 1 min and final products were cooled 40°C for 30 s. This was performed with a Roche Applied Science light cycler 480. All data were normalised to control negative (unstimulated cells) then quantitative expression was determined by comparing with the reference (β-actin) gene expression level according to the Pfaff method (Pfaffl, 2001). The relative expressions of target genes were presented as fold changes in comparison to control negative.

$$\text{Ratio}=\frac{(E_{target})^{\Delta CT,\ target(Calibrator-test)}}{(E_{ref})^{\Delta CT,\ reference(Calibrator-test)}}$$

(CT=Threshold cycle, PCR E=efficiency).

Gene	Forward primer (5′->3′)	Reverse primer (5′->3′)	Probes
VPAC1	TCCGCCAGCCACTCTATC	GCTCGAGCCTGCACAATC	# 19
VPAC2	CCAGGCACTGGTTGTTGATG	CCCCTGAGCACACCTGATG	TCGGTGGTTGGGTGTGGGCA
PAC1	AATTGCTATAGGAAATAATGCAGTAGC	TCATTCCAGAGTTTAGCACAACC	# 69
β-actin	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG	# 64
Rab3a	AACGAGGAATCCTTCAATGCA	TGGGCATTGTCCCATGAGTA	TGCAGGACTGGTCCACCCAGATCA
CaM1	TGCATTCAGGGCTGATTTATAGAG	AACAAGCTACAAAATGCCAGAAAGA	CCCTTGGCTTCTCCTTCTCCTACTCCCT

Table 2.2: Primers and probes used in the human monocytes.
Primers were designed using Roche Diagnostics software and purchased from Eurofins MWG Operon (Germany) and probes were from Roche Diagnostics, Mannheim, Germany).

2.1.14 Impact of calmodulin on the VPAC1 receptor

To find out the effect of calmodulin (CaM) on the VPAC1 receptor expression, fresh-cultured human monocytes were seeded in 24 well-plates. They were then co-cultured for 2h with either 20 μM of calcium-like peptide 1 as CaM agonist (CALP1)(Tocris Bioscience, UK) or 30 μM CaM antagonist, W-7 (Sigma-Aldrich). The invasion assay carried out as described above (2.1.5) in the presence or absence of VIP (10^{-7} M) for 24h. Cells were removed and the VPAC1 expression was measured by both flow cytometry and mRNA quantification of expression level as described above.

2.1.15 Statistical Analysis

Two-tailed unpaired student's *t* test or one way ANOVA was used to determine significant differences between two designated means at the same time point (LPS and LPS+VIP cultured cells and the same with the bacteria in the presence or absence of VIP). Significance values were determined at $P<0.05$ (95% confidence limit).

In qPCR experiments, by using Graph Pad PRISM (6) software, one way ANOVA followed by Tukey's multiple comparison test was used to determine the significant difference ($P<0.05$) among means of more than two groups. Results were normalised to the reference gene (β-actin) using Pfaffl method by advanced relative quantification (light cycler 480 analysis software). Significant values ($P<0.05$) we referred and fold changed were considered by comparing all treated groups to control negative.

Chapter Three

3. The effect of VIP on human monocyte survival and cytokine production following infection with *S*. Typhimurium or culture with LPS

3.1 Introduction

To date, most models of human sepsis involve murine studies or immortalised human immune cells, such as monocytic THP-1 cells, stimulated with LPS. However, the dynamic interaction of primary human blood monocytes with virulent Gram-negative bacteria is likely to be different to that observed in these models, which essentially investigate endotoxaemia rather than bacteraemia. The general model of disease progression in sepsis proposes two phases. The first phase is characterised by uncontrolled production of inflammatory mediators leading to systemic inflammatory response syndrome (SIRS) (Annane et al., 2005, Davis, 2005, Ward et al., 2008) which leads to acute sepsis and may lead to hypo-perfusion and organ collapse (termed septic shock). The second phase of disease is characterised by the production of a compensatory anti-inflammatory response syndrome (CARS), required to restore homeostasis but which can lead to secondary, nosocomial, infection (Gullo et al., 2005, Danikas et al., 2008). The SIRS (acute) phase of sepsis is associated with high systemic concentrations of pro-inflammatory cytokines released by monocytes and macrophages, such as TNF-α, IL-1 and IL-6 (Danikas et al., 2008) and a progression towards uncontrolled systemic inflammation. Thus, intervention which reduces the inflammatory

immune response, has been proposed as a rational therapeutic avenue. However, clinical trials in which this has been attempted have largely failed. One reason is that some agents used may be neutralised by the immune system or degraded by enzymes (Shankar-Hari et al., 2012). Trials investigating inhibition of TNF-α and IL-1β were also disappointing, possibly due to high systemic concentrations of other cytokines and in some cases it seems that the cytokine status of patients was not measured prior to administration of specific cytokine inhibitors (Cohen et al., 2009). Broad ranging anti-inflammatories such as glucocorticoids are also widely used in the treatment of sepsis but their effect is debatable and this may be due to the timing of therapy, dosage and the development of 'steroid resistance' by glucocorticoid receptors (Antonucci et al., 2014).

However studies, to date, have suggested that VIP is an excellent therapeutic candidate for the treatment of sepsis because (i) it is a natural product and does not induce immune response, (ii) it is identical in all mammals apart from guinea pigs (Du et al., 1985), (iii) it is easily synthesised in large quantities, (iv) in murine models of LPS-induced sepsis, low concentrations of VIP (<5 nm) prevent mortality (Delgado et al., 1999a) and (v) VIP inhibits LPS-induced cytokine production in human THP-1 cells (Foster et al., 2005a, Foster et al., 2007a, Foster et al., 2007b). These studies, therefore, suggest that VIP may have significant potential as a cost effective therapeutic against Gram-negative sepsis.

Monocytes respond quickly to bacterial infection via inflammatory chemokines released in infected peripheral tissues (Faix, 2013). Monocytes were used extensively in the past to study sepsis (Xiu and Jeschke, 2013) and LPS-induced disease (Lyakh et al., 2000). We have recently reported on the effect of VIP on the production of inflammatory mediators by human monocytes (rather than monocyte-like THP-1 cells) infected with virulent Gram-negative bacteria and how this compares to the effect of VIP when these monocytes are stimulated with LPS (Askar et al., 2015). This latter study is the basis for the work described in the chapter.

3.2 Material and methods

3.2.1 Isolation of blood monocytes

Human blood was purchased from the blood transfusion service. The blood was diluted with sterile PBS then gently aliquoted onto Histopaque-1077 prior to isolation of the buffer coat, as stated in chapter 2.1.2. After appropriate washing steps, buffy coat supernatants were re-suspended with appropriate amounts of cold MACS buffer and anti-CD14 antibody coated micro-magnetic beads according to the manufacturer's instructions. The viability of isolated monocytes was assessed using Trypan blue (10% v/v) and was found to be > 90 % prior to use.

The cultured monocytes were firstly washed with sterile PBS and the bacteria were added to the cultured monocytes at an MOI of 10:1 and then cultured at 37 °C in Co_2 (5 % v/v) for 60 mins. The cells were

then washed and co-cultured with RPMI media containing 100 µg/ml of gentamycin with or without VIP (according to experimental group) and placed in an incubator for a further 60 mins at 37 °C in Co_2 (5 % v/v). The cultured cells were washed again and the media was substituted with RPMI containing 25 µg/ml of gentamycin with or without VIP (for a further 6 or 24h. The cells were then washed three times with PBS at room temperature and lysed by culture in 1 % Triton-X in PBS for 15 mins at 37 °C. Intracellular bacterial counts were determined by serial dilution at different time points of 2, 6 and 24h post infection. Viable bacterial cells counts were measured as colony forming units per ml (CFU/ml).

In other experiments, monocytes were cultured with LPS, with or without VIP (10^{-7}M), prior to isolation of supernatants for cytokine measurement or cell harvesting.

3.2.2 Stimulation of human monocytes with LPS and VIP

S. Typhimurium 4/74 and LPS from *S*. Typhimurium SL1181 were used to stimulate human monocytes in the studies reported within this chapter. In all experiments, S. Typhimurium 4/74 was cultured with monocytes at a multiplicity of infection of 10:1 (MOI =10). Monocytes were cultured with LPS at a concentration of 100ng/ml, unless otherwise stated.

3.2.3. Monocyte survival assay

The uptake of the fluorescent restriction dye Propidium iodide (PI) was used to measure the viability of cells under the experimental procedures described above. After 24h post-culture monocytes were incubated in PBS containing PI (10 µg/ml) for 10 mins. The number of non-viable cells (PI +) was assessed using a FACSCanto II analyser. Samples were acquired using BD FACSDiva™ and analysed using CyFlogic 2.8 software, licensed to Nottingham University. Monocytes which had been immersed in ice cold (-20 0C) methanol for 30 mins were used as a positive control and monocytes cultured in media only for 24h were used as a negative control. All experiments were performed in triplicate on 3 separate occasions.

3.2.4 Measurement of cytokines using enzyme-Linked immunosorbent assay (ELISA)

ELISA kits were used to measure IL-1β, IL-6, TNF-α, IL-10 and IL-4 in the supernatants isolated from monocytes after 2, 6 and 24h post-infection with *S*. Typhimurium 4/74 or post-culture with LPS, with or without co-culture with VIP. Supernatants isolated from monocytes cultured in media only were used as a negative control. All experiments were carried out in triplicate on 3 separate occasions.

3.2.5 Statistical Analysis

Two-tailed unpaired student's *t* test or one way ANOVA was used to determine significant differences between two designated means at same time point. Significance values were determined at $P<0.05$

(95% confidence limit). All data were analysed and presented by prism software.

3.3 Results

3.3.1 Purity of monocytes

Following Buffy coat and MACS isolation of blood cells the population was analysed for monocyte purity by the shape of the nucleus of isolate cells (via immunofluorescence microscopy) and by measuring CD14 expression and forward scatter (FSC) vs side scatter (SSC) by FACS analysis. This method of isolation was in accordance to several recent studies that used anti-CD14 antibody coated micromagnetic beads for purification of human monocytes, where they obtained 80-95% purity (Kuppner et al., 2003, Rost et al., 2005, Barreiro et al., 2010, Nakaira-Takahagi et al., 2011, Grasso et al., 2014). Immunofluorescence microscopy revealed that most isolated cells had the classic C-shaped nucleus indicative of monocytes (Fig 3.1A, B and C). Flow cytometry analysis (lower panel) through FL-1 (FITC) channel reveals no expression of CD14 antibody expression in isotype antibody (Fig 3.1D), while high expression of CD14 was measured when the cells were incubated with FITC-conjugated mouse anti-human CD14 antibody (Fig 3.1E). The FSC and SSC analysis shows that the cell population was also indicative of monocytes (Fig 3.1F).

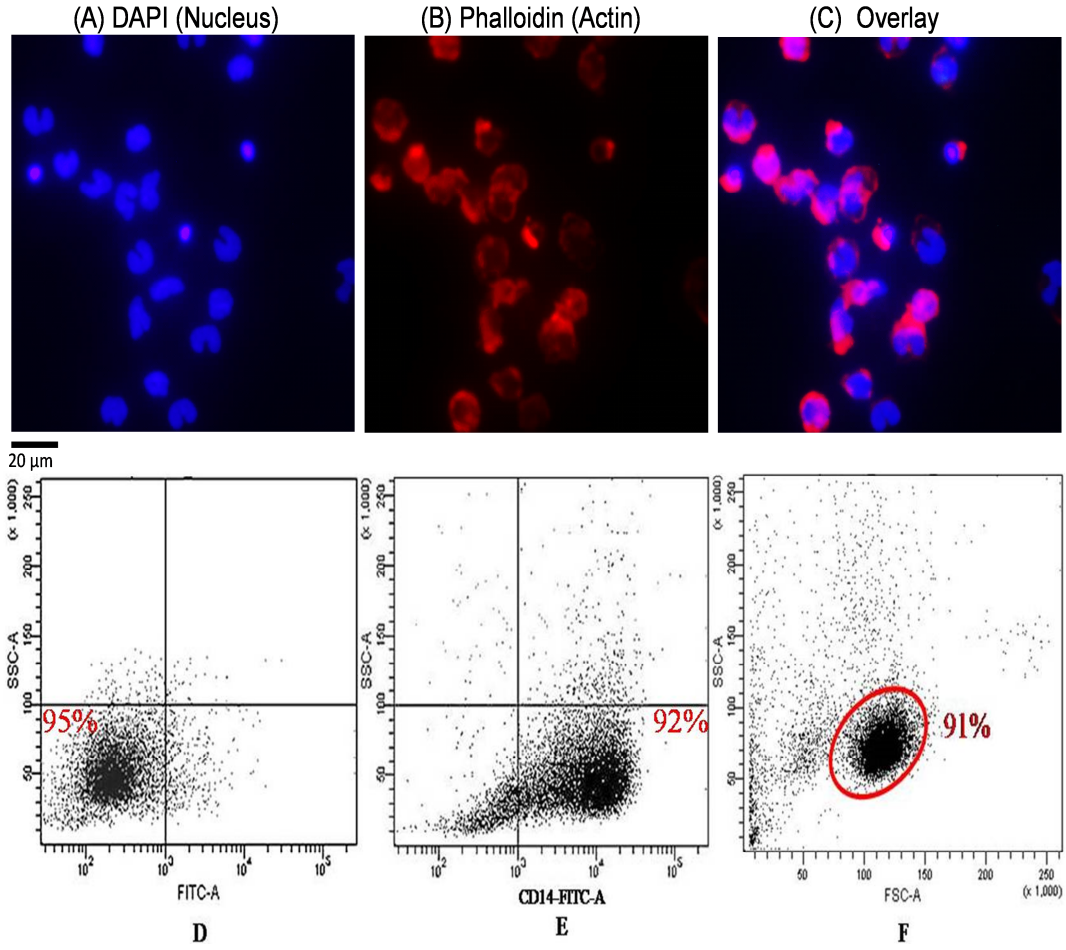

Figure 3.1: Purity of monocytes.

Showing (upper panel) classic 'C' shaped nucleus (A) for most $CD14^+$ cells isolated from buffy coat fraction. B = Cell shape defined by actin localisation. C = Overlay of (A) and (B). Scale bar (Bot tom left) = 20 µm. D= no CD14 expression detected by flow cytometry analysis. E= significantly high CD14 expression when incubated with FITC-conjugated mouse anti-human CD14 antibody. F= shows that the isolated cell population contained >91% monocytes by FSC/SSC analysis.

3.3.2 VIP increases the numbers of *S.* Typhimurium recovered from human monocytes

The results show that VIP (10^{-7} M) was able to enhance intracellular bacterial growth in all time-points as shown (Fig 3.2). However, the potency of VIP was different at different time points. For example, after 2h post-culture, VIP very slightly increased the bacterial growth trend but this was not significantly different (P > 0.05) when compared to the number of *Salmonella* recovered from monocytes which were not co-cultured with VIP (Fig 3.2). After 6h, co-culture of monocytes with VIP induced a significant (P <0.05) increase in the numbers of *Salmonella* recovered compared with monocytes culture with *Salmonella* alone (Fig 3.2). Even after 24h post-culture VIP increased the numbers of *Salmonella* recovered from monocytes compared with monocytes cultured with *Salmonella* alone, but this time point was also not significant (P >0.05) (Fig 3.2).

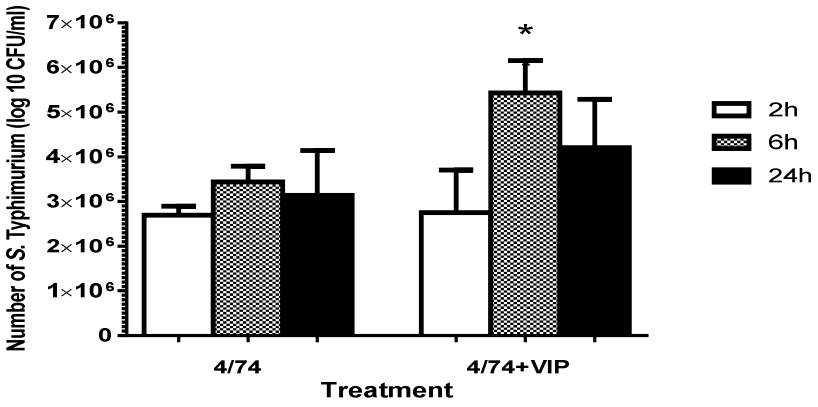

Figure 3.2: Shows the effect of VIP on the growth dynamics of *S.* Typhimurium in monocytes after 2, 6 and 24 h post-culture.

* = significant difference (P <0.05) between the 4/74 co-cultured monocyte in the presence or absence of VIP at the same time-point. Each experiment was performed in triplicate on 5 separate occasions. Bars represent means of SD.

3.3.3 VIP increases survival in human monocytes

The long isolation processes and also interaction of endogenous Fas/Fas ligand may lead to spontaneous apoptosis of human monocyte when cultured *in vitro* (Kiener et al., 1997, Perlman et al., 1999) or by action of ROS (Zhao et al., 2010).

Flow cytometry histogram analysis was used to determine the mortality rate of monocytes. Figure (3.3) is a representative of four different occasions of monocyte mortality. The figure (3.3A) shows that the mean mortality of unstimulated monocytes was 7-12 % when cultured in RPMI media for 24h. Similarly the mean mortality of the monocyte population, when cultured in RPMI media which contained VIP (10^{-7} M) for 24h, was 4% (Fig 3.3B). In contrast, only about 9% of the monocytes survived (91% mortality) when co-cultured with methanol, used as a positive control for mortality (Fig 3.3C). After 24h post-infection (PI) with *S*. Typhimurium 4/74, mortality rate in the monocyte population was 52% (Fig 3.3D) but this was significantly decreased ($P < 0.05$) to 39% when infected cells were co-cultured with VIP (Fig 3.3E). In comparison, when monocytes were stimulated with *Salmonella* LPS (100 ng/ml) for 24h, mortality was quite low 34% (Fig 3.3F) but was further decreased ($P < 0.05$) to 26% when the cells were co-cultured with VIP (Fig 3.3G).

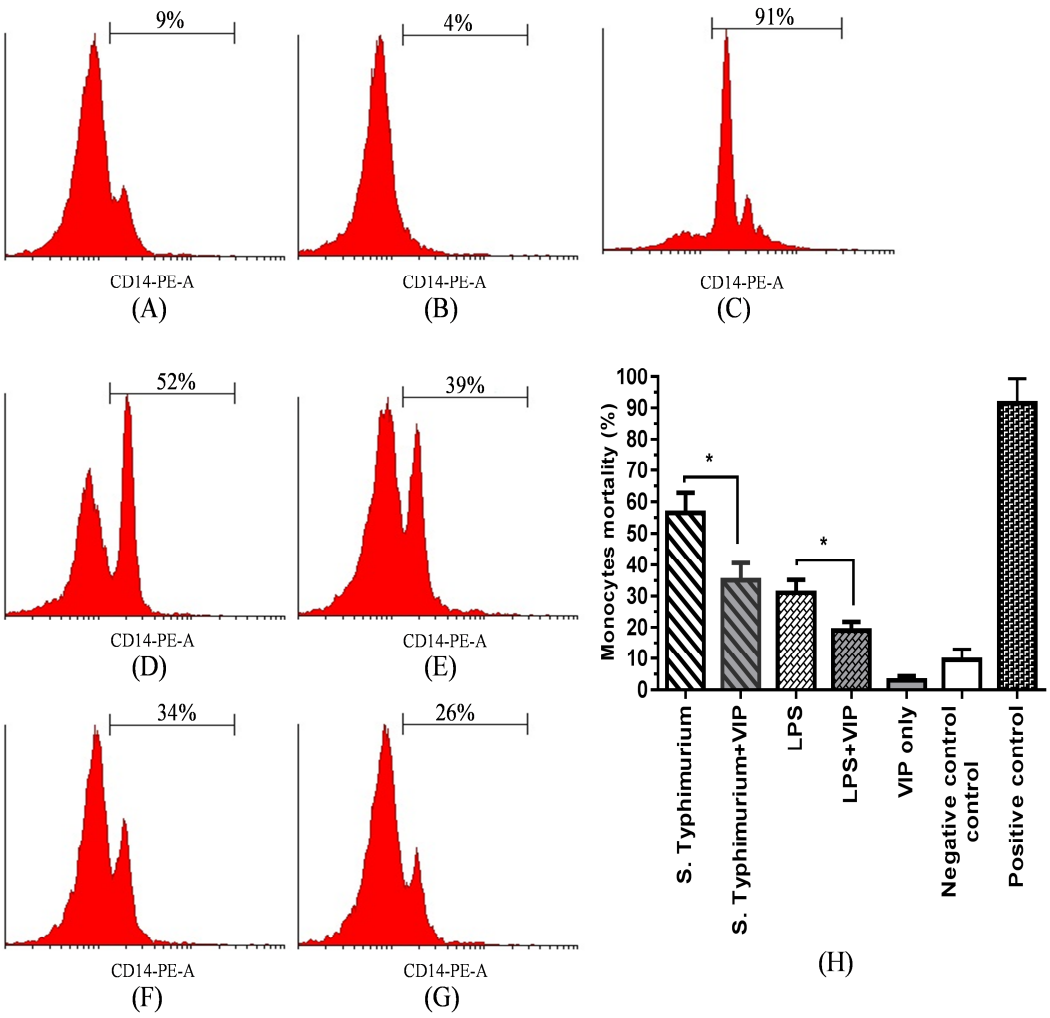

Figure 3.3: Flow cytometry histograms represent the percentage of monocytes death for 24h.

Histogram (**A**) = Untreated monocytes, (**B**) = VIP- treated monocytes for 24h, (**C**) = monocytes cultured with absolute methanol, (**D**) = cultured with *S*. Typhimurium 4/74, (**E**) = monocytes cultured with *S*. Typhimurium 4/74 plus VIP, (**F**) Cultured with LPS and (**G**) with LPS+VIP. (H) Refers to percentage of monocytes mortalities. * = Significant difference P <0.05 between the means of groups in the presence or absence of VIP. Each data is representative of three separate occasions.

3.3.4 VIP reduces the IL-1ß concentration in monocyte supernatants cultured with *S*. Typhimurium or LPS

S. Typhimurium 4/74 increased the concentration of IL-1β in the supernatants isolated from monocytes culture by more than double that measured in supernatants isolated from LPS-cultured monocytes (Fig 3.4). The addition of VIP to the culture media significantly reduced ($P < 0.05$) IL-1β concentrations in the supernatants isolated from *Salmonella*-infected monocytes after 6 and 24h PI (Fig 3.4A). LPS had a much more rapid effect on monocytes and significantly increased cytokine levels within 2h, whereas similar increases were not measured in supernatants isolated from *Salmonella*-infected monocytes at this time point. VIP also reduced the concentration of IL-1β in the supernatants isolated from monocytes cultured with LPS for 6 and 24h, but this was only significant at 24h ($P < 0.05$) (Fig 3.4B).

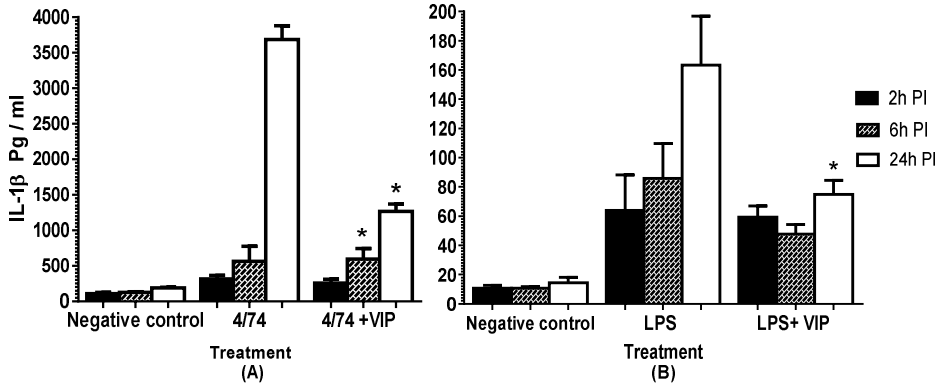

Figure 3.4: VIP inhibits IL-1β production by monocytes cultured with *S*. Typhimurium 4/74 or LPS.

(**A**) = IL-1β concentration in supernatants isolated from monocytes co-cultured with *S*. Typhimurium 4/74, with or without VIP. (**B**) = IL-1β concentration in supernatants isolated from monocytes co-cultured with LPS, with or without VIP. * = Significant difference ($P < 0.05$) between the means of groups in the presence or absence of VIP at the same time-point. Each mean is calculated from triplicate experiments performed on 3 separate occasions. A triplicate performed on 3 separate experiments. Bars represent means of SD.

3.3.5 VIP reduces IL-6 concentration in monocytes supernatants cultured with *S*. Typhimurium or LPS

S. Typhimurium 4/74 significantly increased the concentration of IL-6 measured in supernatants isolated from monocytes after 2, 6 and 24h PI (Fig 3.5A). Co-culture of cells with VIP and *Salmonella* decreased the concentration of IL-6 in supernatants but this decrease was only significantly different ($P< 0.05$) after 24h culture (Fig 3.5A). However, LPS induced a greater and much more rapid IL-6 response after 6h (Fig 3.5B) compared to IL-6 production by *Salmonella*-infected monocytes, although this was equivalent after 24h. Conversely, VIP induced a significant decrease in IL-6 production by monocytes cultured with LPS after 6 ($P <0.05$) and 24h ($P <0.05$), respectively (Fig 3.5B).

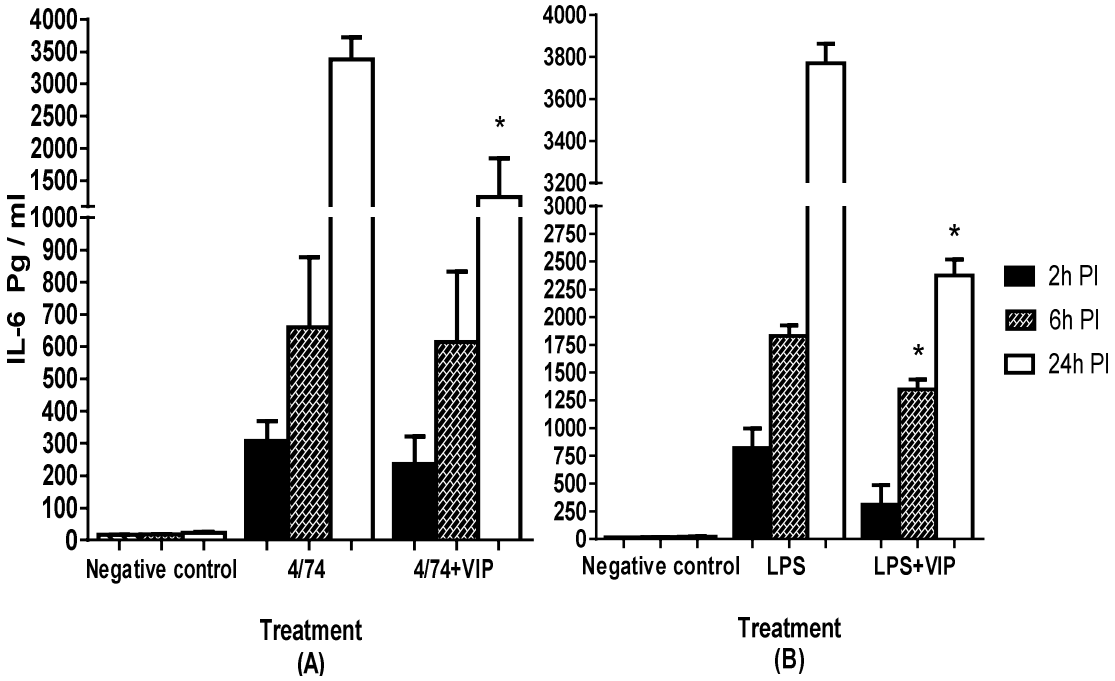

Figure 3.5: VIP inhibits IL-6 production by monocytes cultured with *S.* Typhimurium 4/74 or LPS.

(**A**) = IL-6 concentration in supernatants isolated from monocyte co-cultured with *S*. Typhimurium 4/74, with or without VIP. (**B**) = IL-6 concentration in supernatants isolated from monocytes co-cultured with LPS, with or without VIP. * = Significant difference ($P<0.05$) between the means of groups in the presence or absence of VIP at the same time-point. Each mean is calculated from triplicate experiments performed on 3 separate occasions. Bars represent means of SD.

3.3.6 VIP reduces TNF-α concentration in monocyte supernatants cultured with *S.* Typhimurium or LPS

Co-culture of monocytes with LPS for 2h, induced a noticeable increase in TNF-α concentration measured in cell supernatants compared with the concentration of TNF-α measured in the supernatants from monocytes stimulated with *S*. Typhimurium strain 4/74 (Fig 3.6A). However, co-culture of monocytes with *S*. Typhimurium strain 4/74 induced a significant increase ($P<0.05$) in TNF-α concentration measured in cell supernatants compared with the concentration of

TNF-α measured in the supernatants from monocytes stimulated with LPS at both 6 and 24h time periods.

Co-culturing of VIP with monocytes treated with *S*. Typhimurium strain 4/74 reduced TNF-α production significantly after 6h post-culture and this reduction remained significant at 24h post-culture ($P < 0.05$). Also, addition of VIP into cell cultures containing LPS depressed the TNF-α production curve. The maximal reduction in value means is at 2h whereas the means reduction in TNF-α concentration was still significant ($P<0.05$) after 24h, as shown in figure 3.6B.

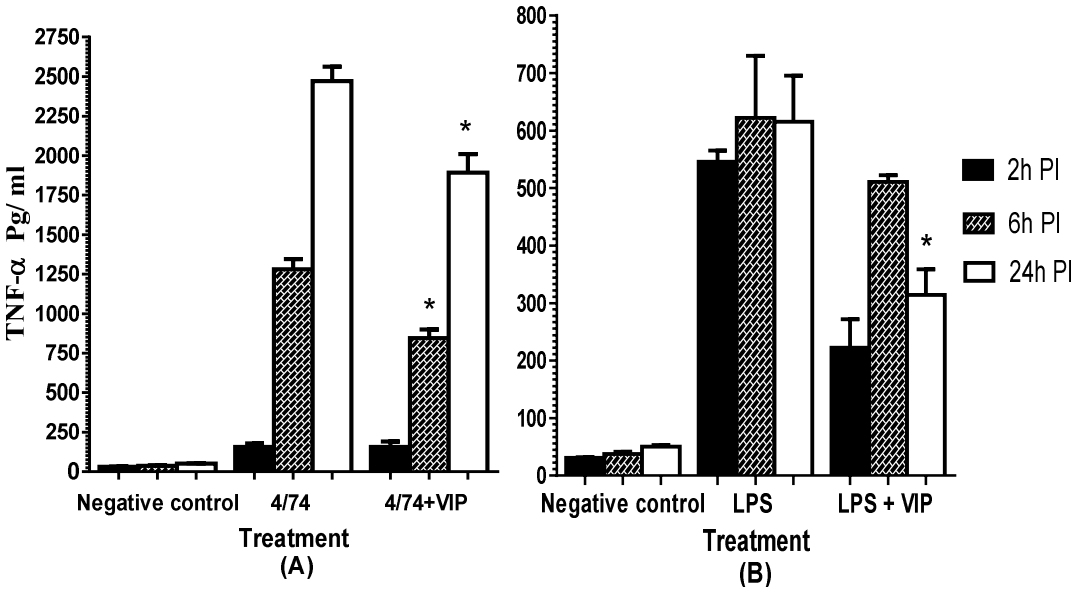

Figure 3.6: VIP inhibits TNF-α production by monocytes cultured with *S*. Typhimurium 4/74 or LPS.

(**A**) = TNF-α concentration in supernatants isolated from monocytes co-cultured with *S*. Typhimurium 4/74, with or without VIP. (**B**) = TNF-α concentration in supernatants isolated from monocytes co-cultured with LPS, with or without VIP. * = Significant difference ($P<0.05$) between the means of groups in the presence or absence of VIP at the same time-point. Each mean is calculated from triplicate experiments performed on 3 separate occasions. Bars represent means of SD.

3.3.7 Effect of VIP on IL-4 concentration in monocytes supernatants cultured with *S*. Typhimurium or LPS

The concentration of IL-4 was also measured, by indirect ELISA, in the supernatants of monocytes which had been cultured with either *S*. Typhimurium strain 4/74 or *S*. Typhimurium LPS in the presence or absence of VIP (10^{-7} M) for 2, 6 and 24h. The results show a significant increase of IL-4 concentration in *S*. Typhimurium treated cells at 2h time course. A further rise in IL-4 level at both 6 and 24h time points also measured. Co-culturing of VIP (10^{-7} M) with *S*. Typhimurium-infected monocytes did not increase the IL-4 concentration significantly ($P > 0.05$) at any of the time points analysed, although after 24h VIP did increase IL-4 production by *S*. Typhimurium-infected monocytes (figure 3.7A).

The effect of LPS on IL-4 production and also the effect of VIP on IL-4 production by LPS-stimulated monocyte were almost identical to that measured in *S*. Typhimurium-infected monocytes (figure 3.7B).

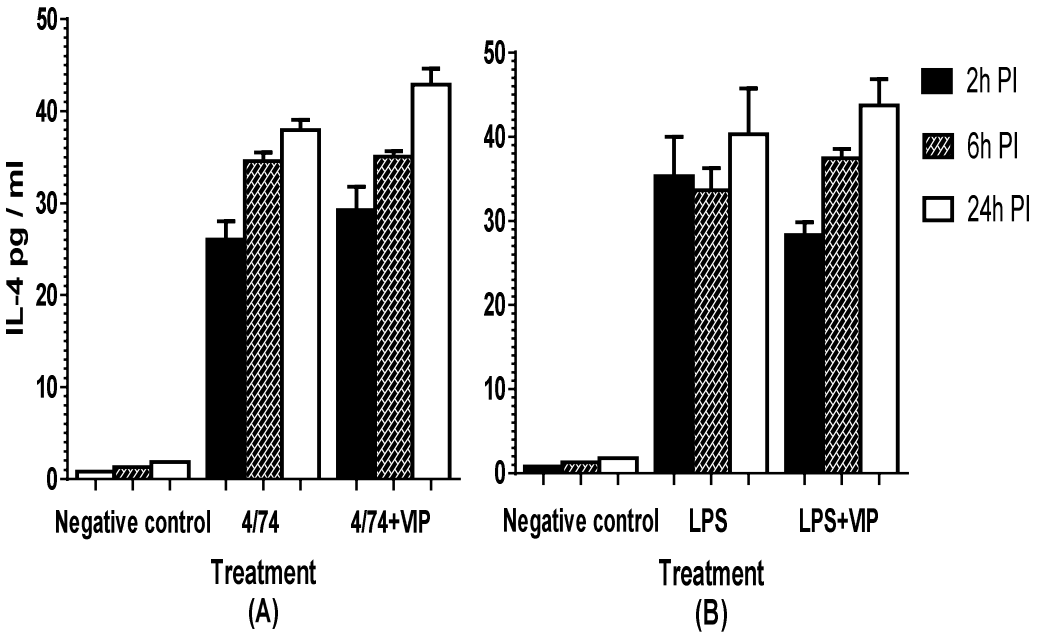

Figure 3.7: VIP effect on IL-4 production by monocytes cultured with *S.* Typhimurium 4/74 or LPS

(**A**) = IL-4 concentration in supernatants of 4/74-treated monocytes, with or without VIP (**B**) = IL-4 concentration in supernatants LPS-treated monocytes with or without VIP. Each mean is calculated from triplicate experiments performed on 3 separate occasions. Bars represent means of SD.

3.3.8 VIP increases IL-10 concentration in monocyte supernatants cultured with *S*. Typhimurium or LPS

The effect of VIP on IL-10 production by LPS-stimulated or *S*. Typhimurium 4/74-infected human monocytes was also measured in this study. The results show that both *S*. Typhimurium infection and LPS-stimulation increased IL-10 production by monocytes (Fig 3.8A and B respectively). Co-culture of *S*. Typhimurium-infected monocytes, or LPS-stimulated monocytes, with VIP (10^{-7} M) also significantly ($P < 0.05$) increased IL-10 production above that measured in the supernatants of cells infected with *Salmonella* (Fig 3.8A) or stimulated with LPS (Fig 3.8B) after 24h. LPS also stimulated increased IL-10 production 6h post-culture but increase was not significant ($P > 0.05$) (Fig 3.8).

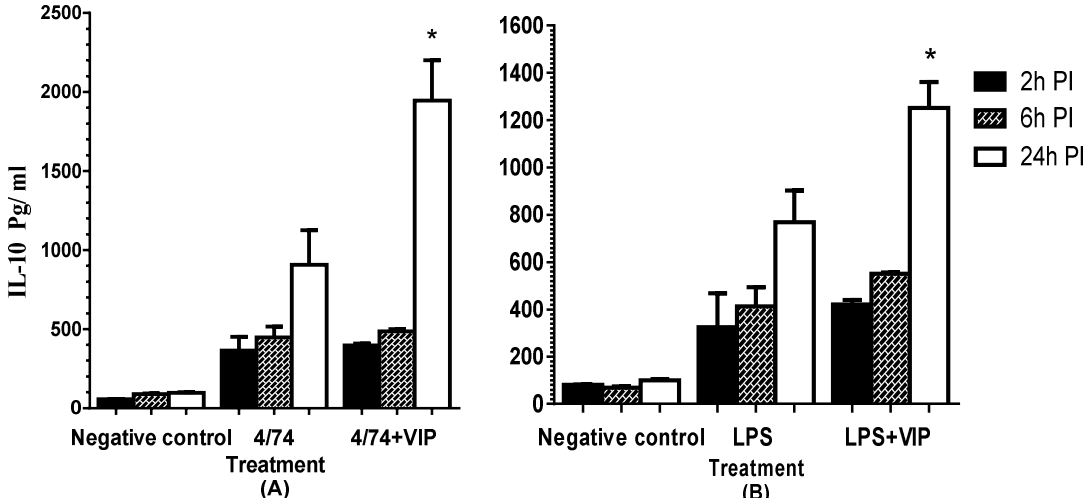

Figure 3.8: VIP increases IL-10 producing by monocytes cultured with *S*. Typhimurium 4/74 or LPS.

(**A**) = IL-10 concentration in supernatants isolated from monocytes co-cultured with *S*. Typhimurium 4/74, with or without VIP. (**B**) = IL-10 concentration in supernatants isolated from monocytes co-cultured with LPS, with or without VIP. * = Significant difference ($P < 0.05$) between the means of groups in the presence or absence of VIP at the same time-point. Each mean is calculated from triplicate experiments performed on 3 separate occasions. Bars represent means of SD.

3.4 Discussion

To date, most studies which have examined the effect of VIP on bacterial-induced inflammatory pathways have involved LPS and either murine macrophages (Delgado et al., 1999e, Delgado et al., 1999d) whole murine models of disease (Delgado et al., 1999a) or immortalised human THP-1 monocytes (Foster et al., 2005a, Larocca et al., 2007, Foster et al., 2007a). Although LPS is a very strong immunogen, it lacks the genetic complexity of intact bacteria. For example, bacteria express a number of other substances such as flagellin, CpG (C-phosphate-G), oligodeoxynucleotides, peptidoglycans and lipoproteins which also initiate an inflammatory response. They also utilise sophisticated secretion systems to invade and survive in cells and which encode proteins that may down-regulate host immune responses.

The ability of *Salmonella* to infect cells, survive and replicate, depends on virulence factors. For example it has been reported that an inability of *Salmonella* to survive inside of murine macrophages renders them avirulent (Fields et al., 1986) which occurs, for example, in *Salmonella* with a mutation in the PhoP/PhoQ regulon (Miller and Mekalanos, 1990). The role of Nicotinamide adenine dinucleotide phosphate (NADPH) and inducible nitric oxide synthase (iNOS) in *Salmonella*-infected mice was studied by Mastroeni et al. (2000). Using iNOS−/−, gp91−/− and *phox*−/− mice challenged with *S.* Typhimurium, the study revealed that NAPDH mediate bacterial killing occurred in the initial stages of infection while iNOS activity mostly occurred in the

latter stages of infection. Thus suggesting that different *Salmonella*-killing pathways may be employed at different times, possibly prolonging the effectiveness of the innate immune response. Murine monocytes infected with *S*. Typhimurium also produce considerable amounts of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-15, IL-18, TNF-α and IFN-γ (Eckmann and Kagnoff, 2001). These cytokines either affect innate immune cells, by promoting innate killing pathways, or the proliferation of Th1 lymphocytes.

There are very few reported studies which have investigated the effect of whole viable bacteria on human monocytes and the role of VIP in this system, as a potential therapeutic in sepsis (Delgado and Ganea, 2003, Smalley et al., 2009, Ibrahim, 2014). In this current study, we show that VIP increased the viability of monocytes infected with *S*. Typhimurium 4/74 or exposed to LPS. The greatest effect of VIP on viability was when monocytes were infected with *Salmonella*, with less than half remaining viable when cultured with *S*. Typhimurium 4/74 for 24h. However, when infected monocytes were co-cultured with VIP, viability increased by >20%. VIP has previously been reported to inhibit LPS-induced cell death in rat neuronal mesenteric cells (Arciszewski et al., 2008) and our study is therefore in accordance with this. *S*. Typhimurium SL1344 has been previously reported to induce apoptosis in human monocyte-derived macrophages (Zhou et al., 2000) and also in the human monocytic cell line, U937 (Forsberg et al., 2003). However, a positive correlation between increased apoptosis of blood monocytes and increased survival of sepsis patients

has been previously reported (Giamarellos-Bourboulis et al., 2006) and elevated monocyte numbers has also been reported to correlate with sepsis (Delano et al., 2011). It is possible, therefore, that the physiological response to sepsis is to reduce some of the capacity of the immune system (monocytes) to produce inflammatory mediators by inducing apoptosis in these cells. If this is the case, then administration of VIP to patients who have Gram-negative bacteraemia may have a detrimental effect. It is probably also the case that the increase in intracellular *Salmonella* survival, following co-culture of cells with VIP, is due to increased monocyte survival. VIP increased intracellular survival of *S*. Typhimurium 4/74 measured at different times post-infection over the 24h period and these results are the basis of our thesis and on recent publication (Askar et al., 2015). These findings are also in accordance with those published by Foster et al. (2005; 2006) which also showed that survival of *S*. Typhimurium 4/74 and 14028 was increased in murine J774 (immortalised macrophages) and was due to reduced oxidative burst (Foster et al., 2006), although intracellular killing pathways were not investigated in this thesis.

Data from human and laboratory animals reveal that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 and are the most significant cytokine produce during sepsis (Blackwell and Christman, 1996, Spittler et al., 2000). In septic patients, IL-6 correlates with serum level of IL-1β and TNF-α (Blackwell and Christman, 1996, Schulte et al., 2013) which may act as biomarkers for the severity and

prognosis of sepsis (Damas et al., 1992, Burkovskiy et al., 2013). In context of TNF-α, the cytokine is the main regulator for the production of inflammatory cytokines (Parameswaran and Patial, 2010) and was regarded as the principal mediator of sepsis (Spooner et al., 1992). TNF-α induces expression of ICAM-1 and VCAM-1 molecules in endothelial cells of septic patients (Nakae et al., 1996, Shimaoka and Park, 2008) and in *E*. Coli-infected baboons administration of anti TNF-α antibody significantly reduced the concentration of IL-1β and IL-6 in the plasma (Fong et al., 1989). IL-1β is another important cytokine produced during many different inflammatory and auto-immune diseases (Dinarello, 2011). In sepsis, IL-1β has been associated with septic shock (Endo et al., 1992), while injection of recombinant-human IL-1β has been associated with sepsis-like clinical signs such as haemodynamic alterations and activation of coagulation cascades in rabbits (Okusawa et al., 1988), primates (Fischer et al., 1992) and humans (Dinarello, 1997).

In this thesis, the effect of VIP on IL-6, TNF-α and IL-1β production by human monocytes infected with *Salmonella* was also investigated. Monocytes infected with *Salmonella* or cultured with LPS produced similar (and significant) amounts of IL-6. While VIP significantly reduced IL-6 after 24h in monocytes infected with *Salmonella*, the effect of VIP was more rapid in monocytes stimulated with LPS (6h). In A study of 203 patients with sepsis, the concentration of IL-6 in plasma was significantly associated with the severity of disease and mortality (Srisangthong et al., 2013). Similarly the ratio of the

concentration of IL-6 and soluble IL-6R has been associated with the induction of septic shock in patients with acute meningococcemia (Pathan et al., 2005). In Askar et al., (2015) we reported that both *S*. Typhimurium 4/74 and LPS increased IL-6R on the surface of monocytes but VIP was only able to reduce this back to steady state levels when the cells were stimulated with LPS and not when infected with *Salmonella*. Taken together these results suggest that VIP may have a greater effect on IL-6 production, and IL-6R expression, when LPS is present in blood rather than when *Salmonella* infection occurs, although free LPS will of course also be present when the blood is infected with *Salmonella*. In the study by Srisangthong et al., (2013) an IL-6 plasma concentration >100 pg/ml was correlated with increased 28 day mortality and results reported here show that, even in VIP co-culture, infected monocytes produce significantly greater concentrations of IL-6 at all-time points measured than this. In an early study by Hack et al. (1989) it was reported that patients with IL-6 plasma concentrations of <40 U/ml IL-6 when administered to hospital with sepsis survived. 1 U/ml of IL-6 is equivalent to 260 pg/ml (Lu et al., 1992) which means that surviving patients in the Hack study had < 10 400 (10.4 ng/ml) IL-6 in their plasma. This level exceeds any level we measured in the monocyte cultures. The study also showed that 89% of the patient cohort with IL-6/plasma concentrations of > 7,500 U/ml (19.5µg/ml) died. This level of IL-6 was not unusual in patients with Gram-negative or Gram-positive sepsis and some patients has IL-6/plasma concentrations which exceeded 100 000

U/ml. It is therefore, difficult to extrapolate our results in the context of *in vivo* studies but it is worth noting that statistical significance *in ex vivo* experiments may not be reflected in biological significance *in vivo*.

Early studies have shown that TNF-α and IL-1β were up-regulated in the blood of volunteers in which LPS was intravenously administered (Hesse et al., 1988, Cannon et al., 1990). When measuring IL-1β in monocyte supernatants, it was found that LPS induced a more rapid and significant effect (after 2h exposure) than was the case for *S*. Typhimurium 4/74, although significant increases in IL-1β were measured in both supernatants after 6 and 24h. The addition of VIP to cultures also differentially affected IL-1β production by monocytes infected with *Salmonella* or stimulated with LPS. In these experiments, VIP significantly reduced IL-1β concentration in the supernatants isolated from *Salmonella*-infected monocytes at both 6 and 24h PI. Although VIP also reduced IL-1β concentration in the supernatants isolated from LPS-stimulated monocytes this was not by a significant amount. It is likely that these discrepancies were due to standard deviations being greater between the replicates used in the LPS experiments, which would suggest that VIP might have significantly reduced IL-1β if more replicate experiments were performed. However, once again whether statistical significance represented biological significance *in vitro* could be questioned. For example, in neonatal sepsis, plasma concentration of IL-1β has been measured at >30 pg/ml and control (non-septic neonates) levels at around 10 pg/ml (Kurt et al., 2008) but it is unlikely that monocytes in these septic

neonates were infected with bacteria at an MOI of 10 or exposed to the concentration of LPS we have used. This can also be said for the results reported above for IL-6 and the results reported below for TNF-α.

TNF-α was the first cytokine to be studied in models of sepsis or in the plasma of septic patients and, following meta-analysis, Lv et al. (2014) reported that TNF-α may be a useful biomarker of neonatal sepsis. Meta-analyses have also indicated that TNF-α therapy may reduce mortality if administered prior to shock, may have therapeutic benefit in patients with high IL-6 titres and that, in paediatric sepsis, TNF-α levels could be used as a biomarker as it strongly correlates with culture of Gram-negative bacteria from septic patients (Kumar and Rizvi, 2009). This latter study may be highly relevant to ours since we show that VIP reduces TNF-α production by 4/74-infected monocytes, which may have therapeutic benefit, although it would also suggest that TNF-α production is a double-edged sword in that it probably is needed to the Gram-negative infection.

However, the reported concentrations of TNF-α in the plasma of septic patients is relatively low, with one study suggesting it could range from 10-100 pg/ml (Schaumann et al., 1997), which may have reflected the different types of infection and the stage of sepsis. In a more recent study, Lekkou et al. (2014) reported that TNF-α concentration was about double that in septic patients compared to non-septic controls over the first 10 days after hospitalisation. Once

again the reported TNF-α concentration was relatively low with a mean of 115 pg/ml on day in septic patients (mortality rate of 44%). Therefore, the concentration of TNF-α (which was associated with sepsis) in these latter studies is much lower than the concentration reported in this chapter which show TNF-α concentrations greater than 115 pg/ml even after 2h exposure to LPS or *Salmonella* (with or without VIP), while after 24h these concentrations were > 600 pg/ml in the supernatants of LPS-stimulated monocytes and >2500 in *Salmonella*-stimulated monocytes.

The production of some anti-inflammatory cytokines, and the effect of VIP on these, was also measured in this study. Since we show that VIP inhibits IL-6 but increases IL-10 production by monocytes it is difficult to say what effect this may have *in vivo* and it is also possible that this could be beneficial or detrimental depending on the phase of disease (SIRS or CARS). A study by (Gogos et al., 2000) reported that IL-10 concentration was positively correlated with the onset of sepsis and that high IL-10/TNF-α ratio indicated a poor prognosis. High circulating levels of IL-6 and IL-10 have also been associated with mortality in other studies (Christ-Crain and Opal, 2010).

We also measured IL-4 production in supernatants isolated from human monocytes challenged with either LPS or *Salmonella*. Interleukin-4 is an important Th2 cytokine which also stimulates differentiation of Th0 cells into Th2 cells and production of IL-10 by murine macrophage (Schmidt-Weber et al., 1999). Also it has been

shown to that IL-4 down-regulated the gene expressions of TNF-α and IL-1β while 2 folds enhanced the LPS-induced IL-Ra production in human monocytes (Vannier et al., 1992). In this chapter, it is shown that supernatants isolated from samples from *Salmonella*-infected monocytes had elevated IL-4 concentrations as early as 2h PI. The level slightly increased at 6 and 24h but was relatively low when compared to other cytokines measured. IL-4 also differed from other cytokines in that the concentration produced by *Salmonell*a-infected monocytes was comparable to the concentration produced by LPS-stimulated monocytes. This trend was not shown by other cytokines which tended to be produced in highest concentration by monocytes infected with *Salmonella* rather than stimulated with LPS. Results in this chapter show that VIP had no effect on IL-4 production, although VIP has been shown to reduce production of both IL-4 in thymocyte T cells (Xin et al., 1997) and to increase IL-4 in murine macrophages (Delgado et al., 2000b) and immature dendritic cells (Delgado et al., 2004). It is possible that the contradictions shown in this study and those previously reported will be explained by different cell types, species and possibly by differences in the concentrations of VIP used.

In conclusion, results in this chapter suggest that VIP inhibited the production of inflammatory cytokines and cytokine receptors by blood monocytes during endotoxaemia, although the timing of VIP intervention (during the SIRS or CARS phase of disease) may be critical. Furthermore, VIP increases the viability of infected monocytes which may have an overall detrimental effect during

bacteraemia/sepsis. Therefore, we suggest that VIP would probably need to be administered as an adjunctive therapy to antibiotic treatment rather than a 'stand-alone' therapy as suggested by earlier murine studies using LPS.

Chapter Four

4. Impact of S. Typhimurium, LPS or VIP treatment on VPAC receptors in human monocytes

4.1 Introduction

The biological action of VIP occurs via three receptors, namely; VPAC1, VPAC2 and PAC1 (reviewed by Harmar et al., 1998). In human resting monocytes, VPAC1 is constitutively expressed heterogeneously (El Zein et al., 2006, Storka et al., 2013). VPAC1 has been shown to be the most important receptor by which VIP exerts an immunomodulatory effect in murine macrophages stimulated with LPS (Delgado et al., 2004) and in murine models of LPS-induced endotoxaemia (Delgado et al., 2000a). In one of the few human studies of its type, Storka et al., (2013) reported an increase in expression of VPAC1 expression by lymphocytes and monocytes following bolus administration of *E*. Coli LPS into human volunteers. In contrast to this result, our study also showed that VPAC2 and PAC1 expression remained unaltered.

Functional studies have also indicated that VPAC1 is the most dominant of the three VIP receptors during endotoxaemia. For example, VPAC1 agonists (K15, R16, L27) significantly decreased nitrite ion production and IL-12 (by about 75%) in the serum of mice in which VIP had been administered (Delgado et al., 1999d, Delgado et al., 1999c). These agonists also inhibit IL-8 production by human THP-

1 cells following LPS culture (Delgado and Ganea, 2003). In another study by Leceta et al. (2000) it was also shown that in murine macrophages and RAW cells, stimulated with LPS, the inhibitory effect of VIP on TNF-α, nitric oxide, IL-6 and IL-12 production also occurs via VPAC1.

Expression of VPAC2 varies depending upon the type of immune cells. It is exclusively expressed in human mast cells (Kulka et al., 2008) while induced by LPS stimulation in murine raw 264.7 cells (Martinez et al., 1998) and peritoneal macrophages (Herrera et al., 2009). Immunohistochemistry showed that VPAC2 expressed at infected region 2 days after detection of VPAC1 in murine alveolar macrophages (Kaltreider et al., 1997).

PAC1 receptor is constitutively expressed in human monocytic THP-1 cells (Delgado and Ganea, 2001b) and murine peritoneal macrophages (Pozo et al., 1997, Delgado et al., 1999d). The receptor induces an anti-inflammatory role in the endotoxic shock by inhibiting IL-6 production and reducing immune cells recruitment (Martinez et al., 2002). By using of PAC1 agonist (5 nM), it has shown that PAC1 abolished over 55% of human immunodeficiency virus type 1 (HIV-1) replication in human monocytes-derived macrophages compared to control negative (Temerozo et al., 2013).

The aim of the work described in this chapter was to examine both the expression and localisation of VPAC1, VPAC2 and PAC1 in human

monocytes challenged with either *S*. Typhimurium or LPS in the presence or absence of VIP.

4.2 Material and methods

Freshly purified human monocytes were stimulated either with *S*. Typhimurium (10:1 MOI) or LPS SL1181 (100 ng/mL) with or without VIP (10^{-7} M) for 24h, as stated in chapter 2.

4.2.1 Expression of VIP receptors by conventional and quantitative PCR analysis

VPAC expression was carried out using Superscript III one step qPCR with platinum Taq. Samples were placed into Bioer XP thermal cycle for the RNA amplification process. The amplification program was set according to the manufacturer's instructions. DNA samples were mixed well with loading buffer then inserted into casting tray (1% gel agarose). To run the gel, the gel electrophoresis apparatus was switched on with 100 volts for 45 mins. Finally, the DNA samples were visualised by UV light, gel photographing was performed using Image Quant 300 imager.

Regarding the relative expression of VPAC receptor by qPCR, briefly RNeay plus kit according to manufacture instruction purified total RNA samples from the cultured human monocytes. The amount and quality of total extracted RNA were measured by NanoDrop and 100ng of each RNA specimen was converted into complementary DNA according to the SuperScript™ First-Strand Synthesis kit's guidance. The PCR reactions were consisted of Light Cycler 480 Probes Master, primer and

universal probe library. The amplification and expression detections were performed by light cycler 480 instrument. Data were analysed using light cycler 480 software, all samples were normalised to control negative according to Pfaff method.

4.2.2 Localisation of VIP receptors in the cell membranes and cytoplasm of monocytes

FACS analyses were used to analyse expression of VIP receptor proteins, as stated in chapter two. Briefly, monocytes were fixed in paraformaldehyde and half of the cells were permeabilised using Triton x-100 in BSA. Subsequently, the cells were washed and re-suspended with primary and with fluorochrome-conjugated secondary antibodies of appropriate species and dilution factors as in table 2.1. The cells were centrifuged and rinsed. VIP receptor was then measured on the cell membrane (non-permebilised cells) and in the cytoplasm (permeabilised cells) using a FACScan ^{TM} flow cytometer system. Data was then acquired using CellQuest pro-software and analysed using FACSDiva software. All results were compared against immunofluorescence signals obtained from appropriate isotype control antibodies.

4.2.3 Statistical analysis

One way ANOVA was used to determine significant differences between means of treated cells. Significance values were determined at $P<0.05$ (95% confidence limit). All data were analysed and presented by prism software.

4.3 Results

4.3.1 Expression of VPAC receptor mRNA

4.3.1.1 Conventional expression of VPAC receptor mRNA

Conventional PCR detected a constitutive expression of VPAC1 whereas very faint detection of VPAC2 as well as PAC1 in untreated human monocytes. VPAC1 further up-regulated was determined by incubation of the cells with *S*. Typhimurium for 24h. Bacterial stimulation also up-regulate the expression of VPAC2 and a lesser degree PAC1 (Fig 4.1). Similar to *Salmonella*, LPS also induced expression all VPAC receptors and the PAC1 is the least expressed. Regarding VIP co-culturing, VIP relatively down-regulated in the bacterial-stimulated VPAC, particularly in VPAC2. In sense of LPS, VIP only down-regulated VPAC2 expression whilst no effect observed on VPAC1 and PAC1 (Fig 4.1).

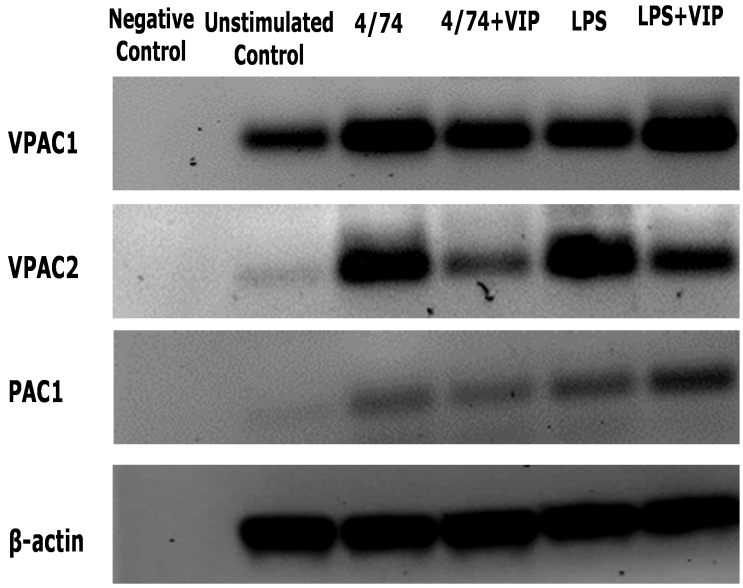

Figure 4.1: Effect of VIP on the VPAC expression levels in the mRNA of human monocytes stimulated with *S.* Typhimurium 4/74 and LPS.

Monocytes were incubated for 24h with *S*. Typhimurium 4/74, Typhimurium 4/74 + VIP, LPS or LPS+VIP. Control negative refers to PCR water instead of cDNA while control positive is housekeeping gene, β-actin. Upper panel is VPAC1, middle panel VPAC2 and PAC1 the lower panel. The experiment was performed on 3 separate occasions.

4.3.1.2 Quantitative expression of VPAC receptor mRNA

4.3.1.2.1 VPAC1

Compared to unstimulated monocytes, VPAC1 mRNA expression in monocytes infected with *S*. Typhimurium 4/74 was significantly ($P < 0.001$) up-regulated but when infected monocytes were co-cultured with VIP (10^{-7} M), VPAC1 mRNA expression was significantly ($P<0.05$) reduced (Fig 4.2). When monocytes were cultured with LPS, for 24h, VPAC1 mRNA expression was also significantly ($P<0.01$) increased when compared to unstimulated cells. However, co-culture of these LPS-stimulated monocytes with VIP also reduced mRNA expression but this was not significant ($P > 0.05$) (Fig 4.2). In contrast to these

results, when monocytes were stimulated only with VIP there was a slight reduction in the expression of VPAC1 (Fig 4.2).

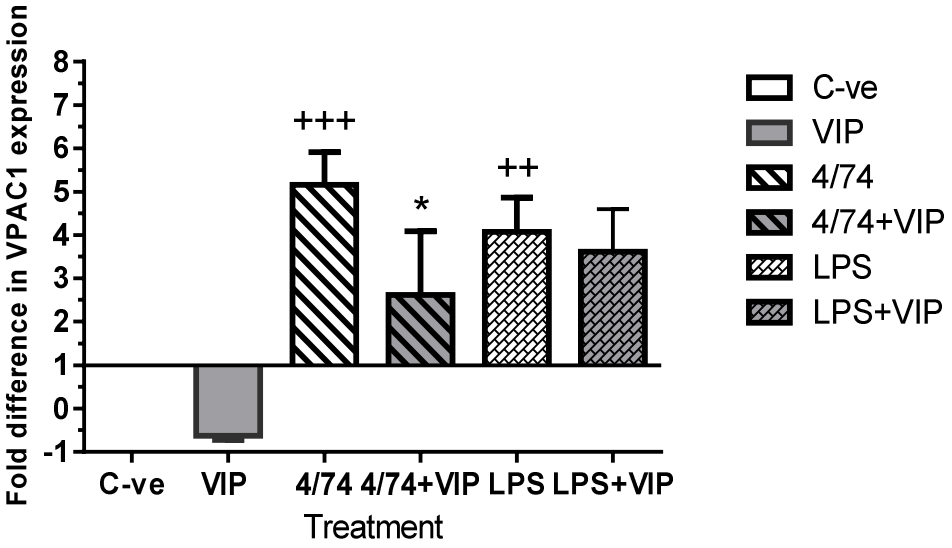

Figure 4.2: Relative expression level of VPAC1 receptor in human monocytes mRNA.

Monocytes were incubated for 24h with either VIP alone (VIP), *S*. Typhimurium 4/74, Typhimurium 4/74 + VIP, LPS or LPS+VIP. Each graph bar represents the receptor relative expression compared to unstimulated cells (control negative, C-ve).* = Significant difference ($P < 0.05$) between VIP-treated cells while + Significant difference ($P < 0.05$, ++= $P < 0.05$, and +++=$P < 0.001$) between bacterial or LPS-treated cells to the untreated. The experiment was performed on 3 separate occasions. Bars represent means of SD.

4.3.1.2.2 VPAC2

VPAC2 expression was highly up-regulated (P<0.001) in *S*. Typhimurium 4/74-treated monocytes in comparison to unstimulated cells. Addition of VIP, slightly reduced *Salmonella*-induced VPAC2 expression but, was not statistically significant at P <0.05.

Similar to the bacterial stimulation, LPS also very significantly expressed VPAC2 (P<0.001) compared to control negative. No obvious effect of VIP was noticed in LPS-expressed VPAC2 compared to LPS co-cultured cells alone. In comparison to unstimulated cells, VPAC2 was slightly down-regulated when the cells incubated with VIP but, was not statistically significant at P <0.05 (Fig 4.3).

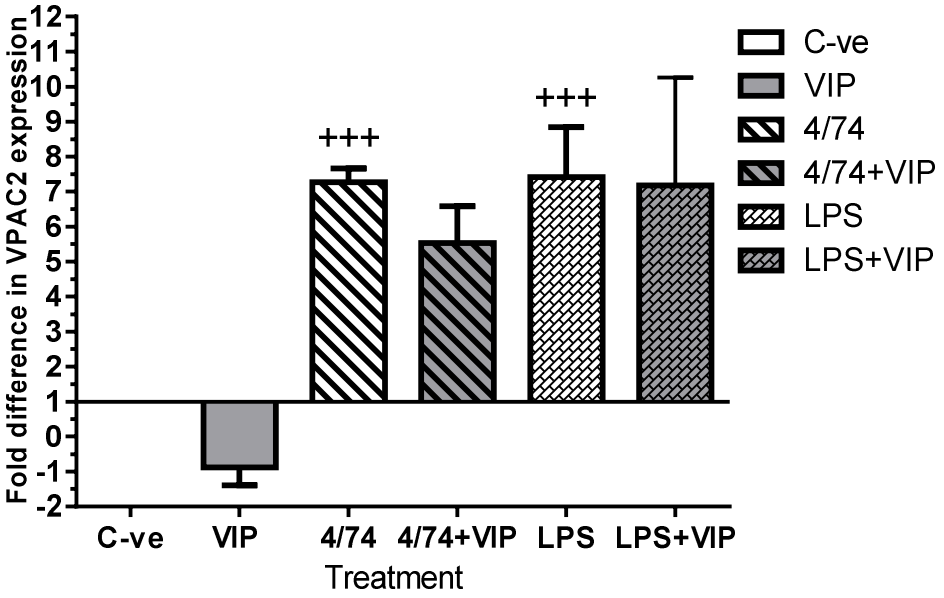

Figure 4.3: Relative expression level of VPAC2 receptor in human monocytes mRNA.
Cells incubated 24h with either VIP alone (VIP), *S*. Typhimurium 4/74, Typhimurium 4/74 + VIP, LPS or LPS+VIP. Each graph bar represents the receptor relative expression compared to unstimulated cells (control negative, C-ve). + = Significant difference (P <0.05 and +++= P <0.001) between bacterial or LPS-treated cells to the untreated. The experiment was performed on 3 separate occasions. Bars represent means of SD.

4.3.1.2.3 PAC1

A very high PAC1 up-regulation was detected in *S*. Typhimurium 4/74-treated monocytes (P <0.01) compared to control negative. When cells were co-cultured with a combination of an *S*. Typhimurium+VIP, a down-regulated PAC1 expression was shown (Fig 4.4) however, this was not significant at P <0.05 compared to the bacterial-treated cells. LPS incubation with monocytes caused a considerable PAC1 expression compared to the untreated one. In cells were co-culture with LPS+VIP a substantial down-regulation of PAC1 was observed compared to LPS stimulated PAC1 (Fig 4.4). Interestingly, VIP-treated monocytes resulted insignificant down-regulation (P<0.05) of PAC1 in comparison to unstimulated cells.

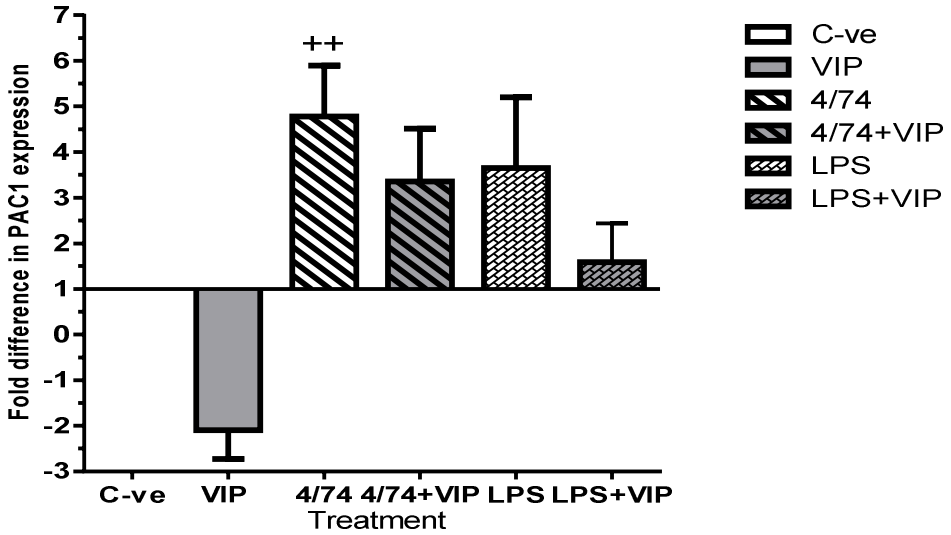

Figure 4.4: Relative expression level of PAC1 receptor in human monocytes mRNA.
Cells incubated 24h with either VIP alone (VIP), *S*. Typhimurium 4/74, Typhimurium 4/74 + VIP, LPS or LPS+VIP. Each graph bar represents the receptor relative expression compared to unstimulated cells (control negative, C-ve).* = Significant difference (p <0.05) between VIP-treated cells other while += significant difference (p <0.05 and ++= p <0.01) between bacterial or LPS-treated cells to the untreated. The experiment was performed on 3 separate occasions. Bars represent means of SD.

4.3.2 Flow cytometric analyses of VPAC surface protein expressions in monocytes

4.3.2.1 VPAC1

The results showed that constitutive expression of VPAC1 protein ($P<0.05$) on the surface of unstimulated (control) monocytes population was about 13% (1300 cells) but when monocytes were cultured with VIP only (10^{-7} M) for 24h, cell population expression was reduced to around 900 (Figs 4.5B and C respectively). In contrast to this, cells expressed VPAC1 increased significantly ($P<0.001$) to almost 4000 on the surface of monocytes which had been infected with *S*. Typhimurium 4/74 for 24h (Fig 4.5D) but when infected monocytes were co-cultured with VIP VPAC1 protein expression decreased significantly ($P<0.01$) to around 2700 (Fig 4.5E). Monocytes cultured with LPS for 24h also showed an increase in VPAC1 expression (to about 2000) when compared to unstimulated controls and this was reduced to 1600 when the LPS-stimulated monocytes were co-cultured with VIP (Figs 4.5F and G respectively). VPAC1 expression in all samples was compared to non-specific expression measured in isotype controls; this was measured as 100 of the total (Fig 4.5A).

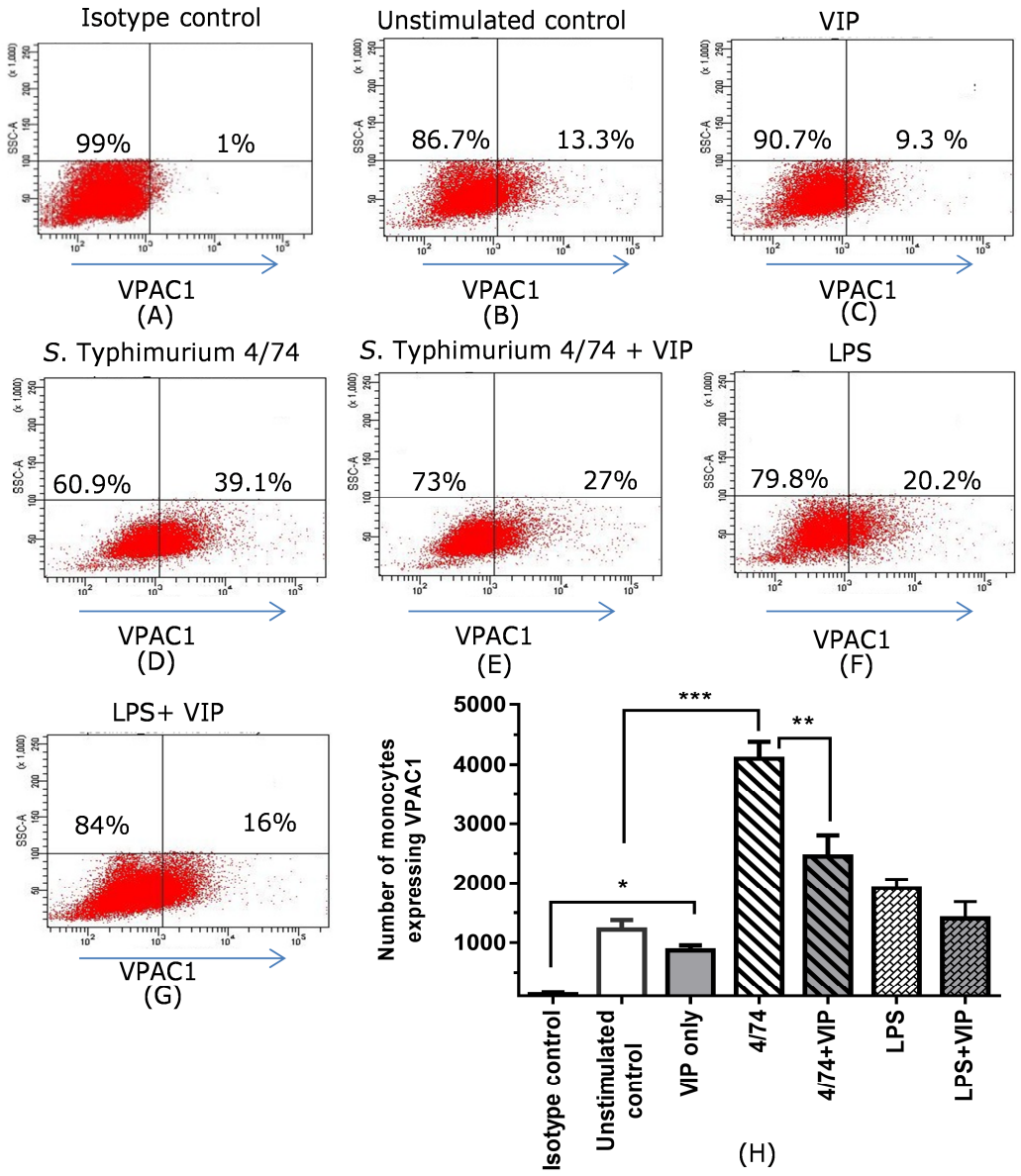

Figure 4.5: Flow cytometric analysis of the expression of VPAC1 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows VPAC1 expression by unstimulated (control) monocytes ;(C) Shows VPAC1 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows VPAC1 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows VPAC1 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows VPAC1 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows VPAC1 expression by monocytes co-cultured with LPS and VIP for 24h; (H) numbers of monocytes expressing VPAC1. * = Significant difference $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.3.2.2 VPAC2

Figure 4.6 shows a modest VPAC2 expression on the surface proteins in human monocytes. Cells populations expressed VPAC2 (780 cells population expression level) in unstimulated (Fig 4.6B) while this rate to substantially increased by addition of VIP for 24h (about 2100, Fig 4.6C). The maximal overexpression in the VPAC2 receptor ($P<0.001$) was seen (Fig 4.6D) on the surface of monocytes co-cultured with *S*. Typhimurium (3700-5000 cells population expression level). No difference was detected in VPAC2 expression between cells co-cultured with *S*. Typhimurium or with *S*. Typhimurium+VIP for 24h (Fig 4.6E). Treatment with LPS also induced the receptor expression significantly ($P<0.05$) to nearly double compare to the unstimulated monocytes (Fig 4.6F) and roughly no change revealed when VIP added to the latter treatment for 24h (Fig 4.6G). VPAC2 expression in all samples was compared to non-specific expression measured in isotype controls; this was measured as 190 of the total (Fig 4.6A).

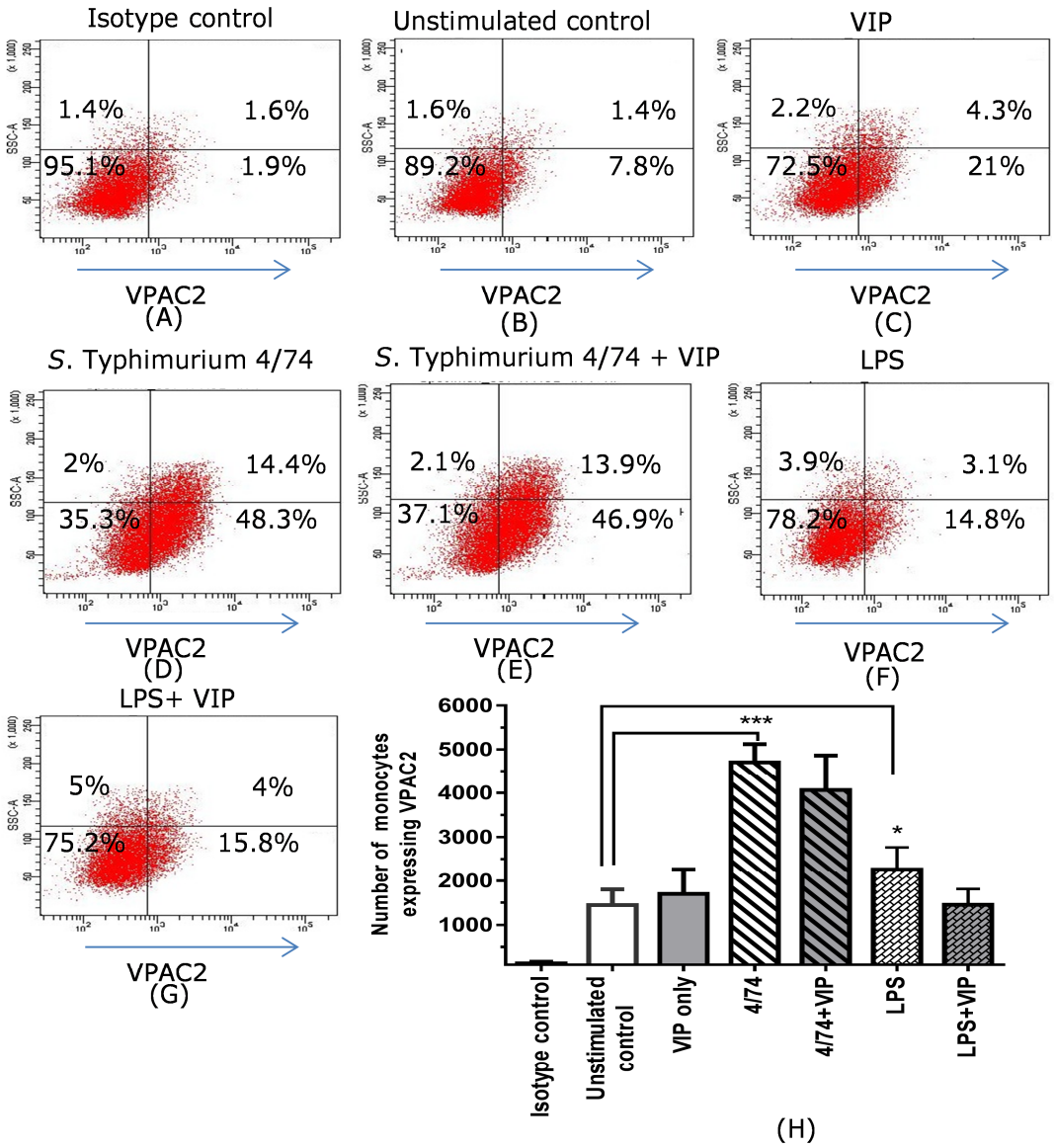

Figure 4.6:Flow cytometric analysis of the expression of VPAC2 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows VPAC2 expression by unstimulated (control) monocytes ;(C) Shows VPAC2 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows VPAC2 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows VPAC2 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows VPAC2 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows VPAC2 expression by monocytes co-cultured with LPS and VIP for 24h; (H) numbers of monocytes expressing VPAC2. * = Significant difference P <0.05, ** = P <0.01 and *** = P <0.001 between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.3.2.3 PAC1

PAC1 expression in all samples was compared to non-specific expression measured in isotype controls; this was measured as 140 of the total (Fig 4.7A). The findings demonstrated a slight expression of PAC1 protein on the surface of unstimulated (control) monocytes population was about 500 cells but when monocytes were cultured with VIP only (10^{-7} M) for 24h, cell population expression was decreased to about 400 (Figs 4.7B and C respectively). In contrary, cells expressed PAC1 raised significantly ($P<0.01$) to around 2300 on the surface of monocytes which had been co-cultured with *S*. Typhimurium 4/74 for 24h (Fig 4.7D) but when infected monocytes were co-cultured with VIP, the PAC1 protein expression reduced to 1900 (Fig 4.7E). Insignificant increase in PAC1 also occurred in monocytes cultured with LPS for 24h (800) in comparison to unstimulated controls. VIP in negligibly reduced this amount (into about 700 cells) when the LPS-stimulated monocytes were co-cultured with VIP (Figs 4.7F and G respectively).

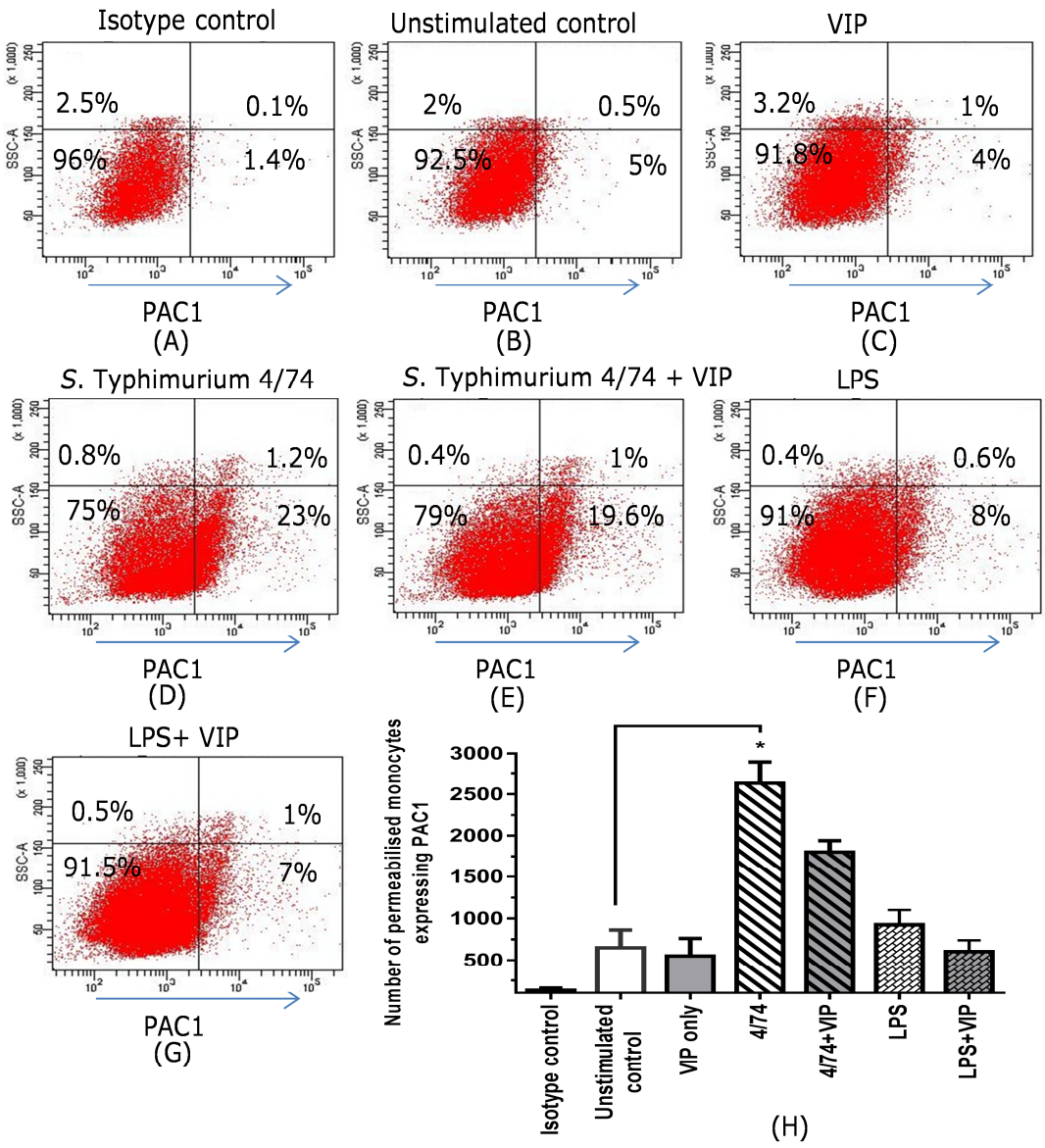

Figure 4.7: Flow cytometric analysis of the expression of PAC1 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows PAC1 expression by unstimulated (control) monocytes ;(C) Shows PAC1 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows PAC1 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows PAC1 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows PAC1 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows PAC1 expression by monocytes co-cultured with LPS and VIP for 24h; (H) numbers of monocytes expressing PAC1. * = Significant difference $P < 0.05$ between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.3.3 Flow cytometry analysis of VPAC intracellular proteins

4.3.3.1 VPAC1

We showed earlier by flow cytometry analysis the expression of surface proteins on all VPAC receptor with different expression intensity. Next, we interested to know whether VPAC receptors have intracellular proteins or molecules that change the expression intensity. Freshly isolated monocytes underwent through all steps like in case of surface protein exposure, however, cells were permeabilised with Triton-x after fixation. The data (Fig 4.8B) shows a constitutive expression of intracellular VPAC1 proteins in the resting human monocytes population which shows significant expression compared to control negative (secondary antibody only) at $P<0.05$. The receptor negligibly reduced (400-700 expression level on positive cells) when VIP was incubated with the cells (Fig 4.8C). In *S*. Typhimurium-treated cells, highly expressed VPAC1 molecules were detected (6500-7500 expression level on positive cells) which contribute the significant expression among all treatments ($P < 0.001$) compared to basal cell expression levels (Fig 4.8D). When VIP co-cultured with the cells treated with *S*. Typhimurium a decreased (about 2000) expression ($P<0.05$) rate was detected by flow cytometry analysis (Fig 4.8E). A significant ($P < 0.05$) VPAC1 expression detected (about 4000) in cells were treated with LPS (Fig 4.8F), and this declined (1000-1600)

substantially but was not significant when VIP added to the cells at P < 0.05 (Fig 4.8G).

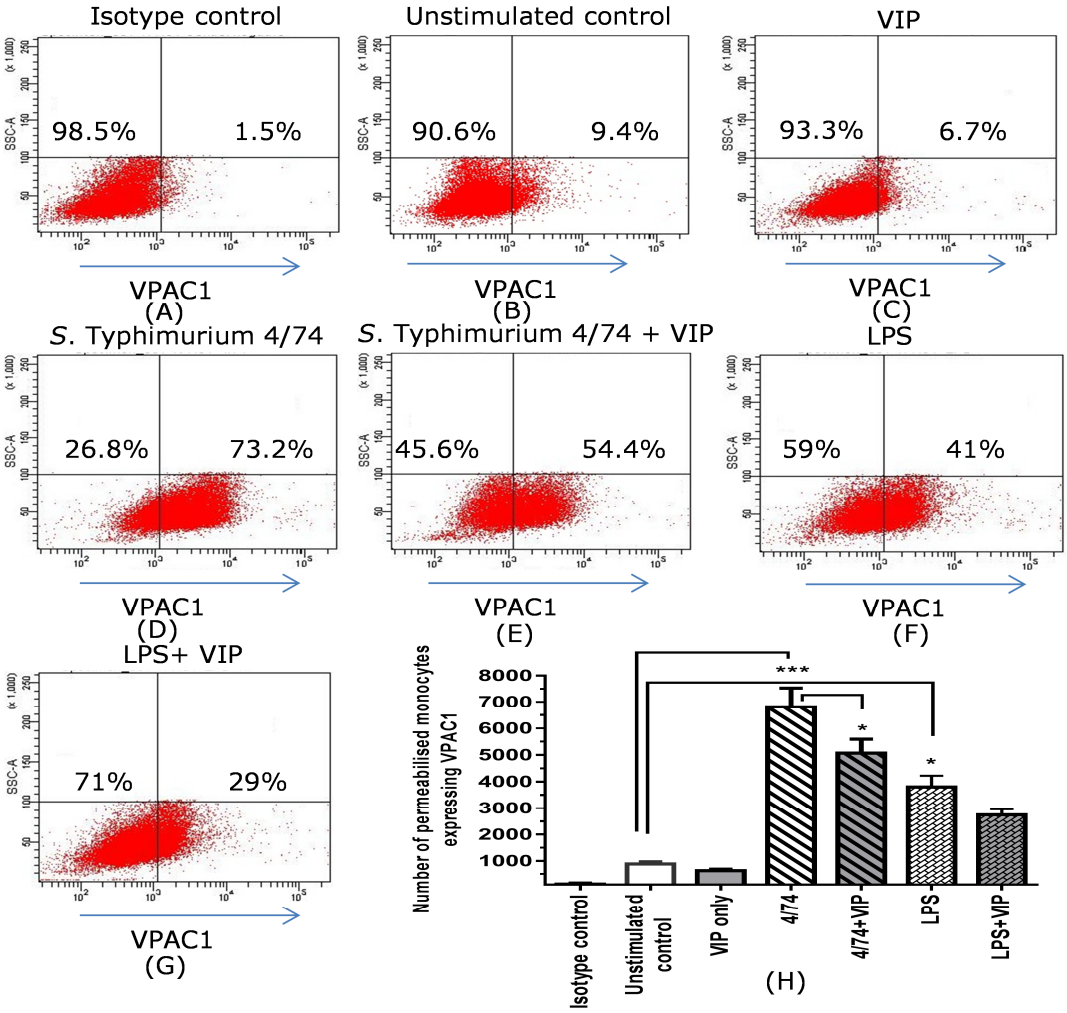

Figure 4.8: Flow cytometric analysis of the expression of VPAC1 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows VPAC1 expression by unstimulated (control) monocytes ;(C) Shows VPAC1 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows VPAC1 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows VPAC1 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows VPAC1 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows VPAC1 expression by permeabilised monocytes co-cultured with LPS and VIP for 24h; (H) numbers of monocytes expressing VPAC1. * = Significant difference P <0.05 and ** = P <0.01 between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.3.3.2 VPAC2

Figure 4.9 demonstrates noticeable expression of VPAC2 intracellular proteins in the resting human monocytes which shows significant expression compared to control negative at P< 0.05 (Fig 4.9B). In contrast to use of VIP in other cases, VIP-incubated cells for 24h slightly enhanced (Fig 4.9C) the VPAC2 expression on cells population (about 300-500). Further increase (P<0.001) of VAPC2 expression molecules (4500-5300 expression level) was found in *S*. Typhimurium-stimulated cells (Fig 4.9D). No apparent changes in the VPAC2 expression revealed after incubation the infected cells with VIP for 24h (Fig 4.9E). Compared to the unstimulated cells, LPS also significantly (P<0.05) enhanced the receptor expression level (about 2200-3000 gated cells, Fig 4.9F) and this declined when VIP added the LPS–stimulated cells (Fig 4.9G) but insignificantly at P<0.05.

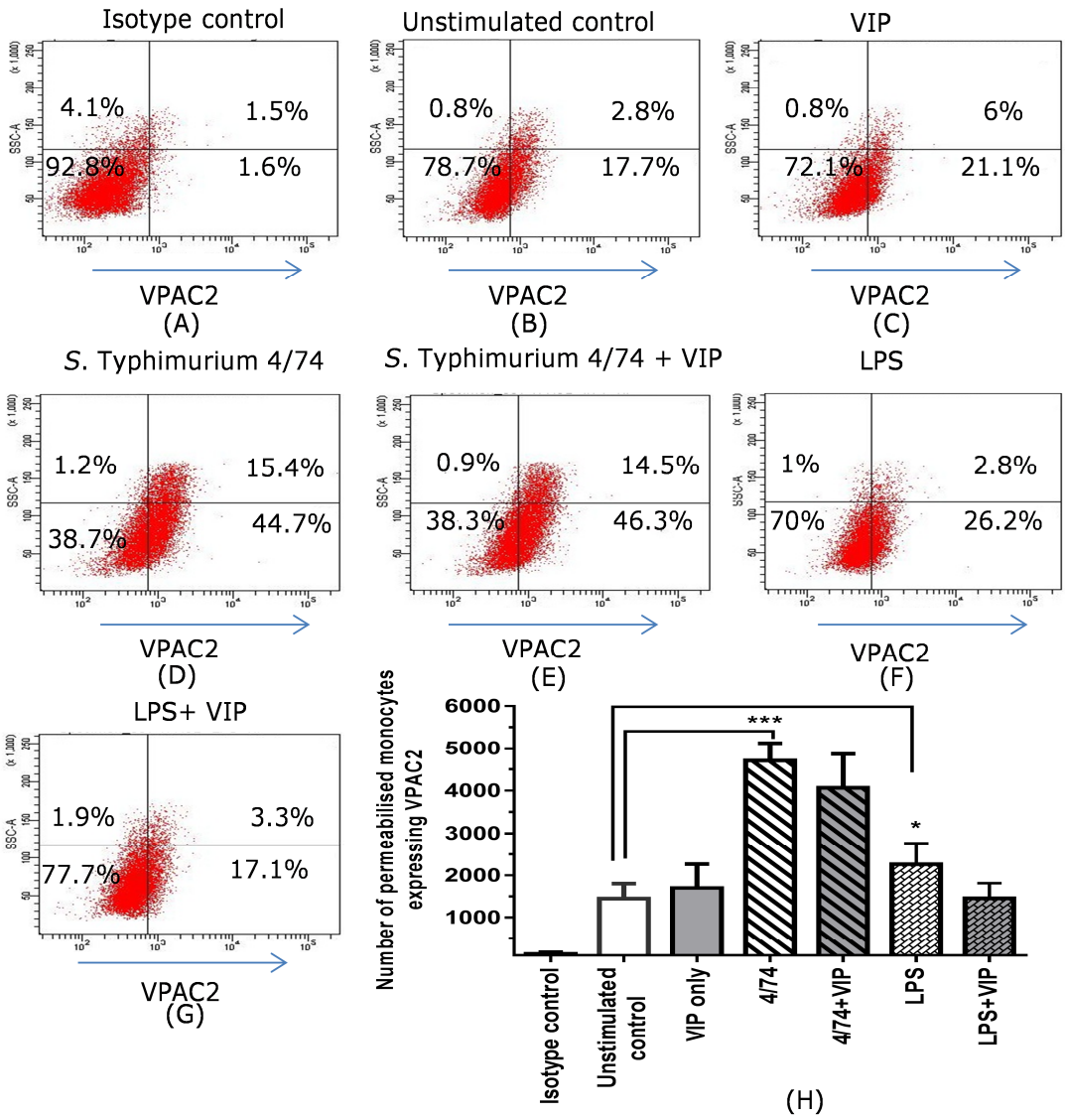

Figure 4.9: Flow cytometric analysis of the expression of VPAC2 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows VPAC2 expression by unstimulated (control) monocytes ;(C) Shows VPAC2 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows VPAC2 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows VPAC2 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows VPAC2 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows VPAC2 expression by permeabilised monocytes co-cultured with LPS and VIP for 24h; (H) numbers of monocytes expressing VPAC2. * = Significant difference P <0.05, ** = P <0.01 and *** = P <0.001 between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.3.3.3 PAC1

The expression of intracellular proteins in PAC1 receptor also was explored by flow cytometric analysis in human monocytes exposed to *S*. Typhimurium, LPS and VIP (Fig 4.10). Results (Fig 4.10B) manifests significant expression of PAC1 expression in the resting human monocytes compared to control negative (secondary antibody only) and when VIP incubated with the cells, it did not alter the expression rate (Fig 4.10C). In *S*. Typhimurium-stimulated cells, a highly expressed ($P< 0.01$) proteins molecule of PAC1 were detected on (about 6600) cell population expression (Fig 4.10D). When VIP incubated with cells treated with *S*. Typhimurium, it reduced the expression rate but was not statistically significant at $P<0.05$ (Fig 4.10E). In cells were stimulated with LPS, a significant ($P<0.01$) increased in PAC1 expression detected (Fig 4.10F) and this dropped slightly when VIP added to 800 cell-expressing PAC1 (Fig 4.10G).

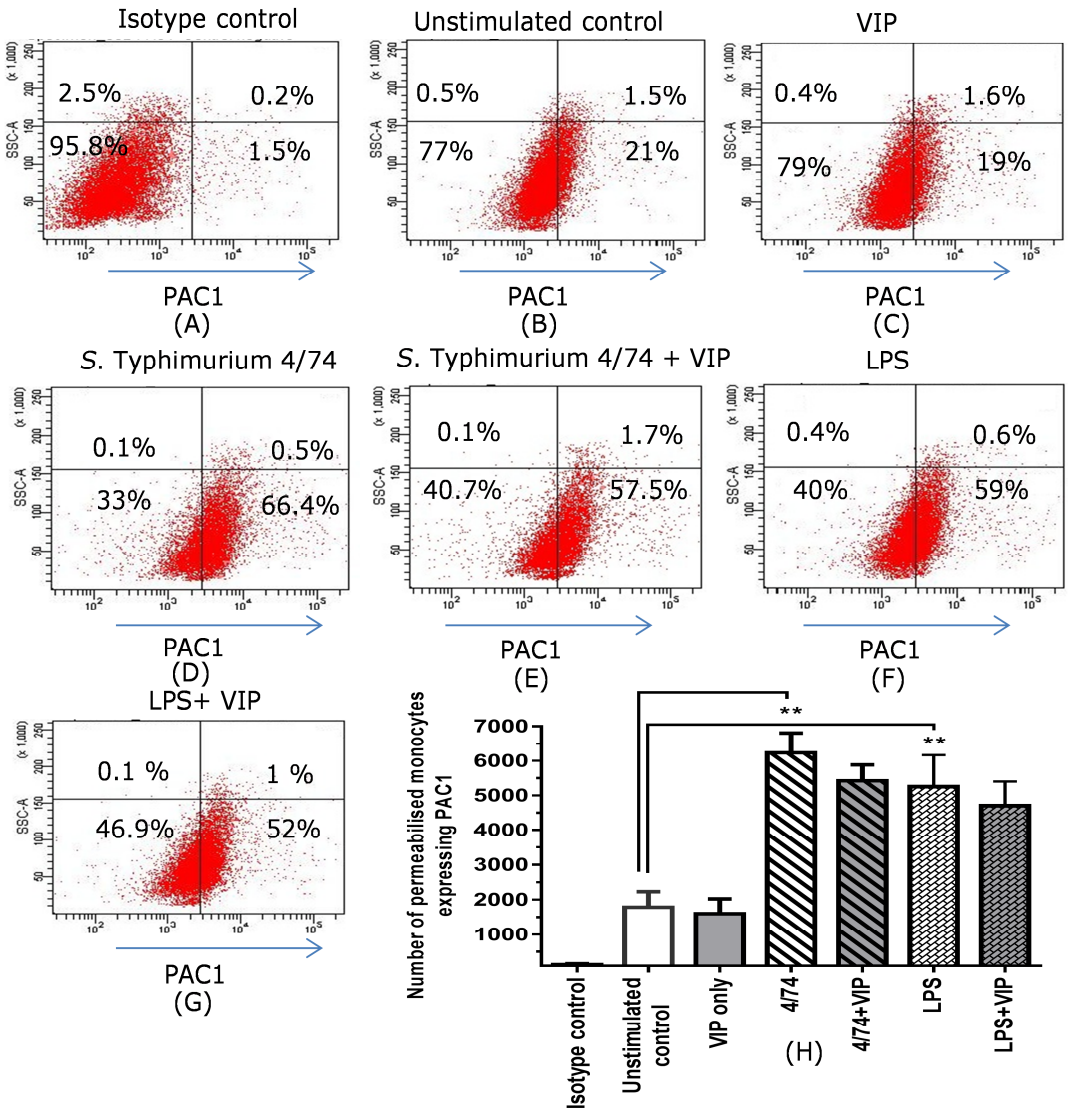

Figure 4.10: Flow cytometric analysis of the expression of PAC1 receptor protein on the surface of human monocytes.

(A) Shows non-specific fluorescence measured from monocytes incubated with a relevant isotype control antibody. (B) Shows PAC1 expression by unstimulated (control) monocytes ;(C) Shows PAC1 expression by monocytes cultured with VIP (10^{-7} M) for 24h; (D) Shows PAC1 expression by monocytes infected with *S*. Typhimurium 4/74 (MOI = 10) for 24h; (E) Shows PAC1 expression by monocytes infected with *S*. Typhimurium and co-cultured with VIP for 24h; (F) Shows PAC1 expression by monocytes stimulated with LPS (100 ng/ml) for 24h; (G) Shows PAC1 expression by permeabilised monocytes co-cultured with LPS and VIP for 24h; (H) numbers of permeabilised monocytes expressing PAC1. * = Significant difference P <0.05 and ** = P <0.01 between the means of groups in the presence or absence of VIP. The data shown is representative of 4 separate analysing performed for each experimental variable.

4.4 Discussion

VIP is a pleiotropic peptide which induces its biological action via three receptors, VPAC1, VPAC2 and PAC1 (Harmar et al., 1998, Lara-Marquez et al., 2001). However, nothing is known about the effect of virulent Gram-negative bacteria, which in addition to LPS also contain a complex array of pathogenicity genes, on expression of VIP receptors in mammalian immune cells. Previous studies have reported that VPAC1 is constitutively expressed by human monocytes (Lara-Marquez et al., 2001, El Zein et al., 2006) and is up-regulated by human monocytes exposed to LPS both *in vitro* (El Zein et al., 2006) and *in vivo* (Storka et al., 2013). The results shown in this chapter also show that VPAC1 is constitutively expressed by resting monocytes at both the mRNA and protein level. Since very little difference was measured between VPAC1 protein expression by permeabilied and non-permeabilied cells, using FACS analyses, this suggested that most of the VPAC1 protein in resting cells was localised to the cell membrane. After 24h PI with *S*. Typhimurium 4/74, VPAC1 mRNA and protein expression increased significantly above that measured in resting cells. This is an interesting finding since the immunomodulatory effect of VIP, as seen by reduced production of pro-inflammatory mediators, has been shown to occur via VPAC1 (Delgado et al., 2001, Fraccaroli et al., 2009). Thus, the results reported here may suggest that *Salmonella* utilise VIP as a means of immunosuppression which would favour intra-cellular survival. In chapter three of this thesis (and in Askar et al., 2015) it was shown that addition of VIP to *S*.

Typhimurium 4/74-infected human monocytes increased survival of the bacteria. Studies by Foster et al., (2005; 2006) have shown this to also be the case in murine J774 macrophages and in these studies VIP was shown to inhibit intra-cellular oxidative killing pathways required to kill the bacteria (Foster et al., 2006), although this was not studied in this thesis nor in Askar et al., 2015. If it is the case that *S*. Typhimurium 4/74 increase VPAC1 expression by monocytes to gain a survival advantage, then it is difficult to explain why the addition of VIP to infected monocytes subsequently decreases VPAC1 expression. It is possible that although the concentration of VIP used in these experiments inhibited pro-inflammatory mediators it was also great enough to cause a negative feedback effect on the receptors. This was not tested during this work and it would be interesting to investigate whether this was the case by culturing monocytes with varying concentrations of VIP and measuring the effect on VPAC1 expression. Another interesting result regarding VPAC1 expression shown in this thesis was the difference between the effect of *Salmonella* and LPS and the effect of VIP on these. The results show that virulent *Salmonella* stimulate a significantly higher expression of VPAC1 by monocytes than does LPS only. VIP also had a slightly reduced inhibitory effect on VPAC1 expression by LPS-stimulated monocytes than monocytes infected with *Salmonella*. Thus, VPAC1 expression must be stimulated by other factors rather than merely LPS (although LPS is still important) and VIP, therefore, must have inhibited the effect of these other factors as well as LPS. Previous studies have reported that

VPAC2 mRNA is not expressed in human monocytes (Lara-Marquez et al., 2001, El Zein et al., 2006). However, the conventional PCR used in this study shows a very low, but detectable, level of expression. A low level of VPAC1 protein expression was also detected on the surface of resting monocytes via FACS analysis. However, higher levels of VPAC2 protein expression were found in the cytoplasm using FACS which has not been investigated in other studies. Addition of *Salmonella* or LPS to monocyte cultures significantly increased VPAC2 mRNA expression, the fold increase was actually greater than that measured for VPAC1 but this can be explained by the high constitutive expression of VPAC1 mRNA and low constitutive VPAC2 mRNA expression. However, unlike the results obtained for VPAC1, VIP reduced VPAC2 expression but not by a significant amount. Similarly, both *Salmonella* and LPS induced a significant increase in VPAC2 protein expression on both the surface and cytoplasm of monocytes but the addition of VIP to these cultures had very little effect. These results may, therefore, suggest that the concentration of VIP required inhibiting VPAC1 expression (the possible negative feedback threshold) is different to that needed to cause the same effect on expression of VPAC2. Previous studies have reported that VPAC2 is expressed when human monocytes (Storka et al., 2013, Hauk et al., 2014) or murine macrophages (Delgado et al., 1999d, Kojima et al., 2005) are exposed to LPS. The studies reported in this thesis, however, are the first to show this effect for virulent *Salmonella* (rather than LPS) and that VIP has little or no effect on expression. What effect increased expression of VPAC2 has in human monocytes

has is not known. Studies using VPAC2 KO mice, and also mice transgenic for VPAC2, have suggested that in $CD4^+$ cells, VPAC2 functions to shift the Th balance towards Th2 (Goetzl et al., 2001, VOICE et al., 2001) but it is not known whether VPAC2 expression and subsequent activation of VPAC2 pathways promotes production of Th2-inducing cytokines by APCs. Conventional qPCR analysis of PAC1 expression did show a very faint band in resting monocytes which may suggest constitutive expression of PAC1. However, this is in contrast to a study by El Zein et al., (2006) which reported that this was not the case but FACS analyses reported in this chapter also clearly show PAC1 protein in the cytoplasm (and to a much lesser extent on the cell membrane) of human resting monocytes. This study also differs to that of El Zein et al., (2006) with respect to up-regulation of PAC1. This latter study reported that LPS did not up-regulate PAC1 mRNA expression in human monocytes, whereas this current study clearly shows, by a number of means, that both virulent *Salmonella* and LPS up-regulates PAC1 at both the mRNA and protein level. This discrepancy may be explained by the fact that El Zein et al., (2006) exposed the monocytes to LPS for only 10 minutes, whereas we used much longer time points. The effect of VIP on PAC1 expression was more similar to that observed for VPAC2, rather than VPAC1, with VIP reducing PAC1 protein expression in infected, or LPS-stimulated, cells by <10 %. Administration of PACAP, acting via the PAC1 receptor, has been shown to inhibit LPS-induced septic shock in mice and is associated with a significant decrease in IL-6 (Martinez et al., 2002).

Thus, it is possible that *Salmonella* also up-regulate expression of PAC1 to inhibit the pro-inflammatory environment (by inhibiting cytokines such as IL-6) which may increase survival advantage.

Chapter Five

5. Internalisation and recycling pathways of the VPAC1 receptor in the human monocytes

5.1 Introduction

Increase calcium concentration has been reported following LPS stimulation. In septic shock, it is found over two folds elevation in the calcium concentration in the rat brain (Anderson et al., 1999) and also in cardiac myocytes of guinea pigs (Thompson et al., 2000). This increase occurred in the early stages, in comparison to non-septic controls, and this may result in several metabolic disorders. Binding of calcium to its sensor protein, calmodulin (CaM), modulates many subsequent targets via both protein phosphatases and kinases (Kapiloff et al., 1991). The most important calcium/CaM effective family is CaMK, which is required for mediating inflammatory reaction (Rosengart et al., 2000). CaMK (type II and IV) blockage suppressed the ERK, JNK, MAPK and AP-1 pathways, induced by LPS (Cuschieri et al., 2003). Furthermore, Kim et al. (2014) suggested that CaMKII binds to IRAK1 and enhances NF-KB activity. In parallel, calcium/CaM can interact with several SNARE proteins, including Rab3a, to control the regulated exocytosis of many hormones and neurotransmitters (Barclay et al., 2005). Role of Rab3a has not been studied in immune cells, whereas in neurons it is implicated in vesicle maturation, mobilisation, and membrane fusion at synaptic junctions (Leenders et al., 2001, Coleman et al., 2007).

Some studies have suggested a significant increase in the concentration of endogenous VIP in murine serum during sepsis (Zamir et al., 1992). Upon VIP/VPAC interaction, VPAC is promptly phosphorylated, which initiates signalling cascades prior to receptor desensitisation which prevents further signalling (McDonald et al., 1998). VPAC2 receptor has been reported to be likely phosphorylated by GRK and PKA and desensitized by arrestin (McCulloch et al., 2002). Arrestin induces signal cascades and then internalise the receptor into clathrin-coated endosomes (von Zastrow, 2003). Endosome production is governed by GTPases, particularly Rab5a which recruits EEA1 that tethers, docks and fuses vesicles to SNARE, which subsequently leads to endosomal shipment (Chia and Gleeson, 2014).

The internalised GPCR cargo is either directed to the late endosome (Maxfield and McGraw, 2004) where it degrades and ceases the receptor signalling (Luzio et al., 2007). Alternatively, the receptor cargo may recycle instantly to the cell membrane, via Rab4 or Rab5, or slowly into recycling endosome via Rab11 (Cheng and Filardo, 2012). Rab11 is localises to the recycling endosome and regulates two-way material exchanges between early endosomes and TGN (Huotari and Helenius, 2011). Nothing is known about the role of Rab3a in human mononuclear cells. In PC12 cells, it was shown that Rab3a is associated to newly formed secretory granules throughout different developing stages (Handley et al., 2007). In addition, Rab3a protein is also found on the newly produced secretory vesicles on the TGN surface of pancreatic acina cells (Jena et al., 1994).

The object of the work described in this chapter was to determine how *Salmonella*, LPS and VIP affect endocytosis and exocytosis of the VPAC1 receptor in human monocytes.

5.2 Materials and methods

5.2.1 Co-localisation of VPAC1

Freshly purified human monocytes were stimulated either with *S*. Typhimurium or LPS in the presence or absence of VIP for 24h. The cells were fixed, permeabilised, blocked and washed in PBS as described in 2.1.8 section. The monocytes were then incubated with primary and secondary antibodies of VPAC1 and an appropriate antibody for detection of some internalisation and recycling-associated markers. Finally, Vectashield Hard-set mounting medium labelled with DAPI and visualised later by confocal scanner laser mounted coverslips onto clean slides.

5.2.2 Effects of VIP on Rab3a and CaM in *Salmonella*-infected or LPS-stimulated monocytes

The effect of VIP on expression of Rab3a and CaM mRNA was investigated in *Salmonella*-infected or LPS-stimulated monocytes after 24h as stated in chapter two.

5.2.3 Impact of CaM on VPAC1 expression

To delineate the effect of CaM on VPAC1 expression in monocytes, LPS firstly stimulated cells or *Salmonella* then were treated with CaM agonist and antagonist. Later, VPAC1 expression was detected by both flow cytometry and qPCR to same methods were stated in details in 2.1.14.

5.2.4 Statistical analysis

One way ANOVA was used to determine significant differences between means of the treated cells in the presence or absence of VIP. Significance values were determined at $P<0.05$ (95% confidence limit). All data were analysed by prism software.

5.3 Results

5.3.1 Internalisation and recycling of VPAC1 receptor in human monocytes

5.3.1.1 VPAC1 protein localisation with the early endosome

In order to investigate the VPAC1 internalisation pathways, association of VPAC1 protein with the early endosome antigen (EEA1) was first studied using confocal microscopy. No immunoreactivity to VPAC1 protein or EEA1 was observed in controls in which monocytes were incubated with secondary antibody (Fig 5.1A). In unstimulated monocytes cultured with both primary and secondary antibodies, weak immunoreactivity to VPAC1 protein was observed in the trans-membrane region of the cell and some co-localisation of VPAC1 protein with EEA1 was also observed within this region (Fig 5.1B). When monocytes were cultured with VIP (10^{-7} M) for 24h, immunoreactivity to VPAC1 protein and EEA1 was similar to that observed in unstimulated cells. Thus, weak immunoreactivity to VPAC1 protein was also observed in the trans-membrane region of the cell and some co-localisation of VPAC1 protein with EEA1 within this region (Fig 5.1C). However, when monocytes were incubated with *S*. Typhimurium 4/74 strong immunoreactivity to VPAC1 protein, with a granular-like distribution pattern within the cytoplasm, and high co-localisation of VPAC1 protein and EEA1 was observed (Fig 5.1D). Co-culturing of *S*. Typhimurium 4/74-infected cells with VIP appeared to reduce the strength of the immunoreactivity to VPAC1 protein but the distribution (within the cytoplasm) was not altered and high co-localisation of

VPAC1 protein with EEA1 was still retained (Fig 5.1E). Similarly, in monocytes stimulated with LPS (100 ng/ml) for 24h, strong immunoreactivity to VPAC1 protein was also observed but this was seen mostly in the transmembrane region of the cell rather than the cytoplasm. However, high co-localisation of VPAC1 protein with EEA1 was also observed within this region (Fig 5.1F). When LPS-stimulated monocytes were co-cultured with VIP, weaker immunoreactivity to VPAC1 protein was observed when compared to monocytes stimulated only with LPS. VPAC1 protein distribution was similar (trans-membrane) and was also co-localised with EEA1 in the transmembrane region (Fig 5.1G).

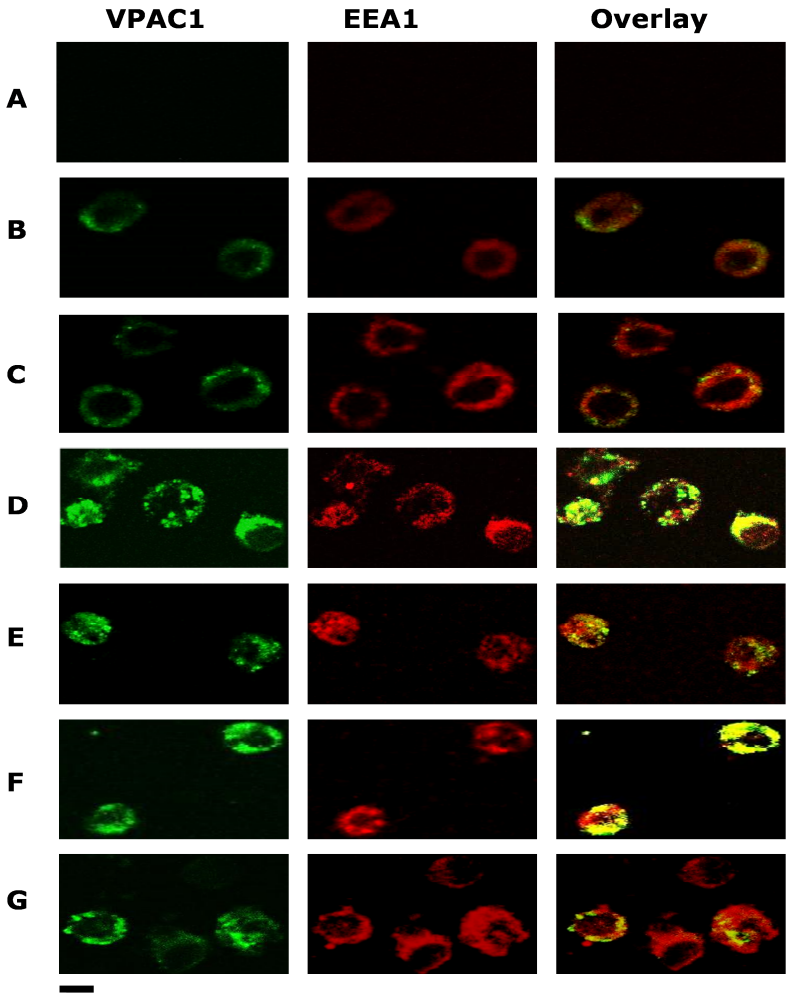

Figure 5.1: Confocal microscopy image showing the comparative immunoreactivity and distribution of VPAC1 protein and EEA1 in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to VPAC1 or EEA1 in control monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, VPAC1 1 and EEA1 in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, VPAC1 and EEA1 in monocytes stimulated with VIP (10^{-7} M) for 24 h. Lane (D) shows the immunoreactivity to, and distribution of, VPAC1 and EEA1 in monocytes infected with S. Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, VPAC1 and EEA1 in monocytes infected with S. Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, VPAC1 and EEA1 in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, VPAC1 and EEA1 in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of VPAC1 and EEA1. Scale bar (bottom left) = 10 μm.

5.3.1.2 VPAC1 localisation with lysosomes

Confocal laser scanning microscopy was also used to investigate whether VPAC1 internalisation occurred within lysosomes as shown by co-localisation with lysosomal membrane associated membrane protein 1 (LAMP1). In unstimulated monocytes cultured with both primary and secondary antibodies, weak immunoreactivity to VPAC1 protein was observed in the trans-membrane region of the cell and some co-localisation of VPAC1 protein with LAMP1 was also observed within this region (Fig 5.2B). No change in LAMP1 immunoreactivity was observed in monocytes was cultured with VIP for 24h (Fig 5.2C). Strong immunoreactivity of VPAC1 protein also insignificant co-localisation with LAMP1 was seen when monocytes were incubated with *S*. Typhimurium 4/74 (Fig 5.2D). Co-culturing of VIP with *S*. Typhimurium 4/74-infected cells reduced the strength of the immunoreactivity to VPAC1 protein but did not affect significantly on the VPAC1 and LAMP1 co-localisation (Fig 5.2E). Similar to *Salmonella*-co-cultured monocytes, strong immunoreactivity of VPAC1 protein was detected in the trans-membrane of LPS exposed cells for 24h. In comparison monocytes stimulated only with LPS, weaker immunoreactivity of VPAC1 protein was observed when these cells co-cultured with VIP (Fig 5.2F). In parallel, VIP reduced co-localisation of VPAC1 and LAMP1 protein this could be due higher distribution of VPAC1 in the transmembrane region of LPS-stimulated cells (Fig 5.2G).

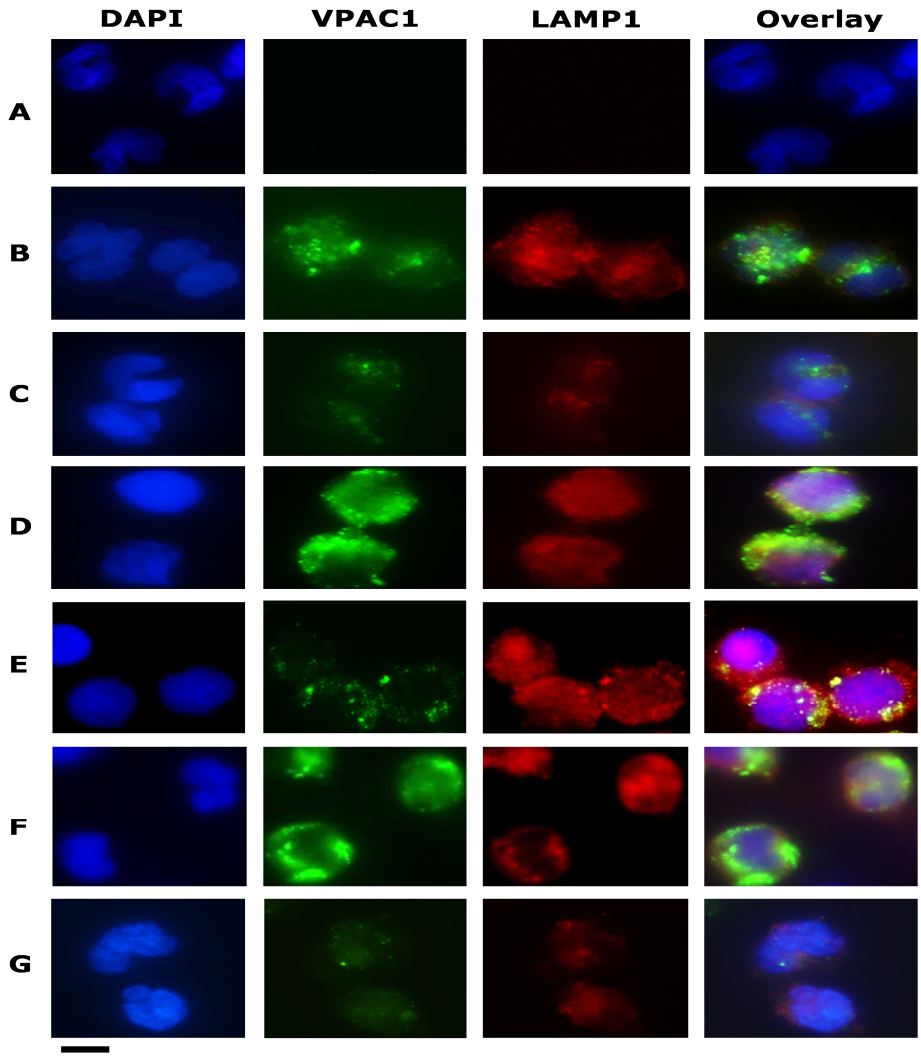

Figure 5.2: Immunofluorescence microscopy image showing the comparative immunoreactivity and distribution of VPAC1 protein and LAMP1 in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, VPAC1 1 and LAMP1 in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, VPAC1 and LAMP1 in monocytes stimulated with VIP (10^{-7} M) for 24h. Lane (D) shows the immunoreactivity to, and distribution of, VPAC1 and LAMP1 in monocytes infected with *S.* Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, VPAC1 and LAMP1 in monocytes infected with *S.* Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, VPAC1 and LAMP1 in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, VPAC1 and LAMP1 in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of VPAC1 and LAMP1. Scale bar (bottom left) = 10 μm.

5.3.1.3 Localisation of VPAC1 protein with the recycling endosome marker Rab11a

Rab11a regulates two-way material exchange between the early endosomes and TGN (Wilcke et al., 2000). To examine whether VPAC1 protein is trafficked from the endosome to the TGN via Rab11a, confocal microscopy was used. No immunoreactivity to VPAC1 or Rab11a proteins was observed in controls in which monocytes were incubated with secondary antibody (Fig 5.3A). In unstimulated monocytes cultured with both primary and secondary antibodies, weak immunoreactivity to VPAC1 protein was observed in the trans-membrane region of the cell and some co-localisation of VPAC1 with Rab11a proteins was also observed within this region (Fig 5.3B). When monocytes were cultured with VIP (10^{-7} M) for 24h, immunoreactivity to VPAC1 and Rab11a proteins was similar to that observed in unstimulated cells. Thus, weak VPAC1 immunoreactivity was also observed in the trans-membrane region of the cell and some co-localisation of VPAC1 with Rab11a proteins within this region (Fig 5.3C). However, when monocytes were incubated with *S*. Typhimurium 4/74, strong immunoreactivity to VPAC1 protein was observed with high co-localisation of VPAC1 and Rab11a proteins (Fig 5.3D). Co-culturing of *S*. Typhimurium 4/74-infected cells with VIP appeared to reduce the strength of immunoreactivity to VPAC1 protein but the distribution (within the cytoplasm) was not altered and high co-localisation of VPAC1 with Rab11a proteins was still retained (Fig 5.3E). In monocytes stimulated with LPS (100 ng/ml) for 24h, strong

immunoreactivity to VPAC1 protein was also observed but this was seen mostly in the transmembrane region of the cell rather than the cytoplasm. High co-localisation of VPAC1 and Rab11a proteins was also observed within this region (Fig 5.3F). When LPS-stimulated monocytes were co-cultured with VIP, weaker immunoreactivity to VPAC1 protein was observed when compared to monocytes stimulated only with LPS. However, VPAC1 protein distribution was similar (trans-membrane) and was also co-localised with Rab11a protein in the transmembrane region (Fig 5.3G).

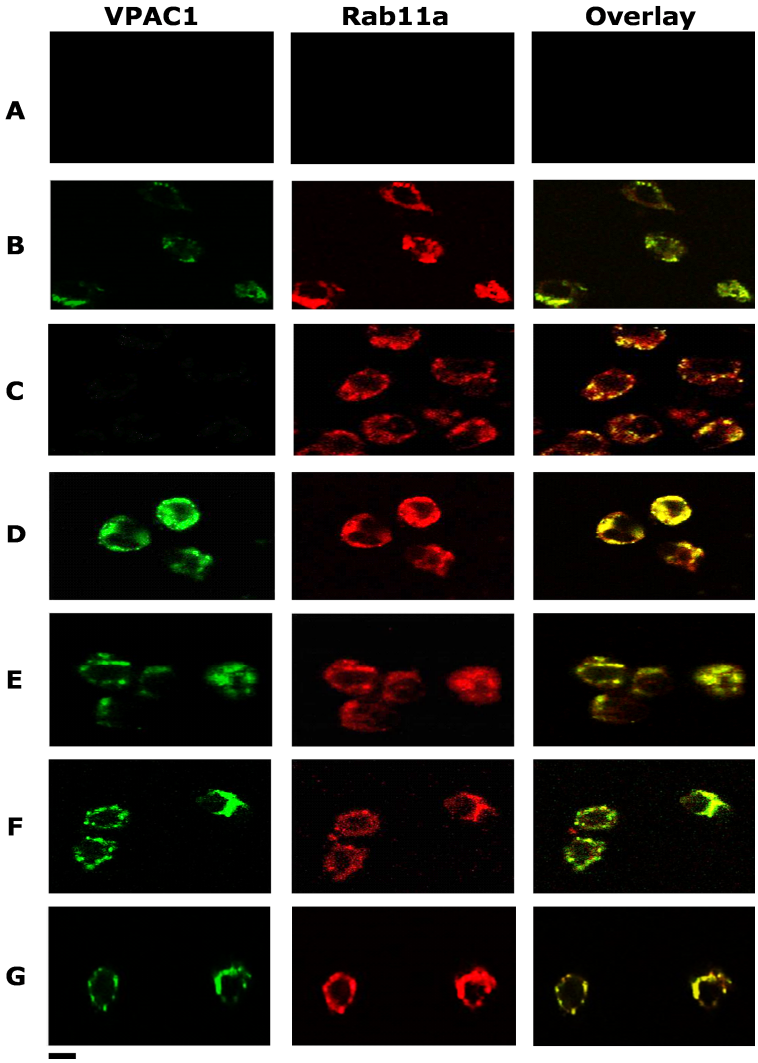

Figure 5.3: Confocal microscopy image showing the comparative immunoreactivity and distribution of VPAC1 and Rab11a proteins in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to VPAC1 or Rab11a in control monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, VPAC1 1 and Rab11a in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, VPAC1 and Rab11a in monocytes stimulated with VIP (10^{-7} M) for 24h. Lane (D) shows the immunoreactivity to, and distribution of, VPAC1 and Rab11a in monocytes infected with *S.* Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, VPAC1 and Rab11a in monocytes infected with *S.* Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, VPAC1 and Rab11a in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, VPAC1 and Rab11a in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of VPAC1 and Rab11a. Scale bar (bottom left) = 10 µm.

5.3.1.4 VPAC1 localisation with the TGN marker (TGN46)

Studies to this point suggested that VPAC1 was transported from endosomes via Rab11a, the next experiments were designed to investigate whether, or not, VPAC1 was associated with the TGN which is known to be the major sorting site of proteins (Wilcke et al., 2000).

Considerable immunoreactivity of VPAC1 protein was detected in the trans-membrane region unstimulated monocytes cultured with both primary and secondary antibodies. Also, some co-localisation of VPAC1 protein with TGN was noticed within this region (Fig 5.4B). In comparison to unstimulated cells, no apparent alteration in the immunoreactivity and also co-localisation was observed as monocytes co-cultured with VIP (10^{-7} M) for 24h (Fig 5.4C). Strong immunoreactivity to VPAC1 protein and high co-localisation with TGN was found in monocytes incubated with *S*. Typhimurium 4/74 (Fig 5.4D). Reduction in immunoreactivity of VPAC1 proteins whereas negligible alteration in VPAC1 and TGN co-localisation was revealed in the bacterial-infected monocytes co-cultured with VIP (Fig 5.4E). Similarly, in monocytes stimulated with LPS (100 ng/ml) for 24h, strong immunoreactivity to VPAC1 protein was also seen mostly in the transmembrane region. However, high co-localisation of VPAC1 protein with TGN was also noticed within this region (Fig 5.4F). Co-culturing of VIP with LPS-stimulated monocytes for 24h, appeared to reduce the strength of VPAC1 immunoreactivity compared to LPS alone. In addition, VIP has an effect on the distribution of VPAC1 on the

cytoplasm of LPS-stimulated monocytes and also lessened VPAC1 co-localisation with TGN in this area (Fig 5.4G).

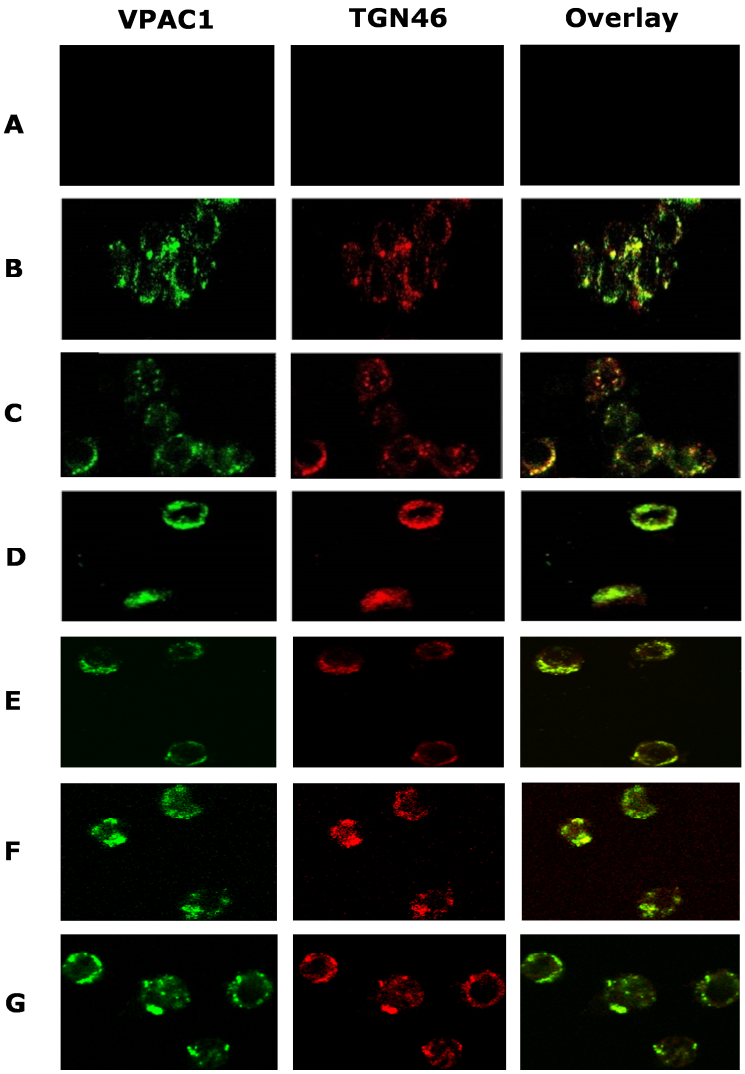

Figure 5.4: Confocal microscopy image showing the comparative immunoreactivity and distribution of VPAC1 and TGN46 proteins in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to VPAC1 or TGN46 in control monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, VPAC1 1 and TGN46 in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, VPAC1 and TGN46 in monocytes stimulated with VIP (10^{-7} M) for 24 h. Lane (D) shows the immunoreactivity to, and distribution of, VPAC1 and TGN46 in monocytes infected with *S.* Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, VPAC1 and TGN46 in monocytes infected with *S.* Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, VPAC1 and TGN46 in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, VPAC1 and TGN46 in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of VPAC1 and TGN46. Scale bar (bottom left) = 10 μm.

5.3.1.5 Localisation of Rab11a protein with the TGN in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP

Previous results in this chapter show that VPAC1 is co-localised to Rab11a and also the TGN. Rab11a protein is located on both the recycling endosome and the TGN and regulates two-way material exchange between early endosomes and the TGN (Wilcke et al., 2000). It was, therefore, important to investigate whether Rab11a was also co-localised with the TGN in human monocytes under the experimental conditions used in this study.

No immunoreactivity to TGN46 or Rab11a protein was observed in controls in which monocytes were incubated with secondary antibody only (Fig 5.5A). In unstimulated monocytes cultured with both primary and secondary antibodies, weak immunoreactivity to TGN46 and Rab11a protein was observed in the trans-membrane region of the cell but co-localisation was apparent (Fig 5.5B). When monocytes were cultured with VIP (10^{-7} M) for 24h, immunoreactivity to TGN46 and Rab11a protein appeared to increase and co-localisation was retained (Fig 5.5C). When monocytes were infected with *S*. Typhimurium 4/74 (Fig 5.5D) or stimulated with LPS (Fig 5.5F) for 24h increased antibody immunoreactivity to both TGN46 and Rab11a protein was observed and in both cases this was reduced by co-culture with VIP (Fig 5.5E and 5.5G respectively).

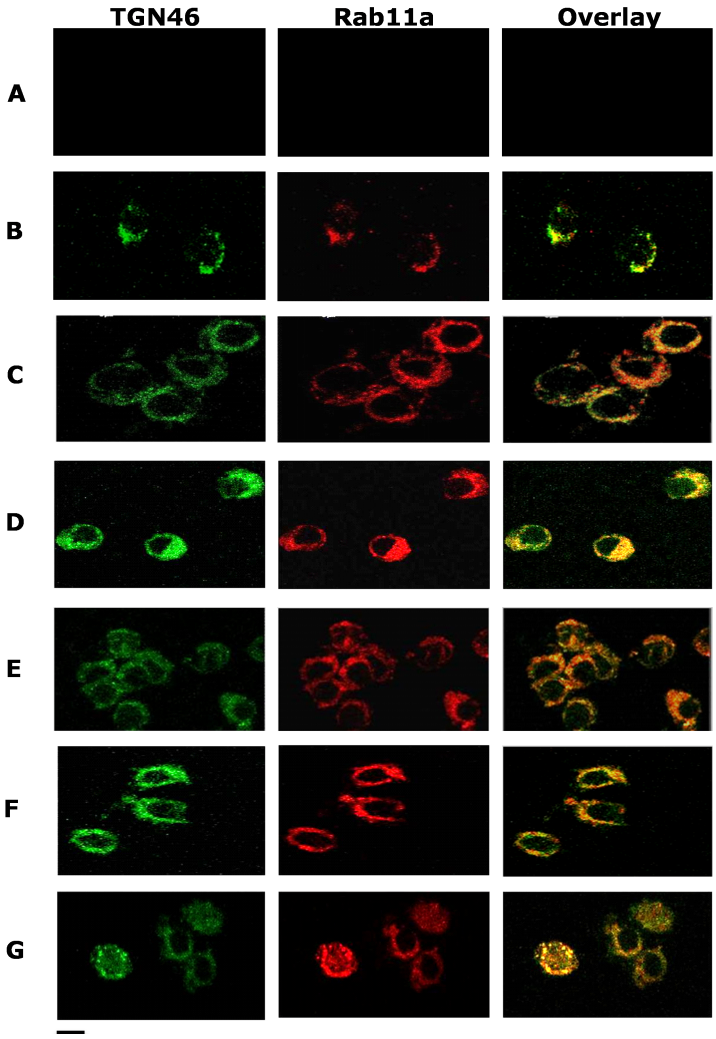

Figure 5.5: Confocal microscopy image showing the comparative immunoreactivity and distribution of TGN46 and Rab11a protein in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to TGN46 or Rab11a in control monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in monocytes stimulated with VIP (10^{-7} M) for 24h. Lane (D) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in monocytes infected with *S.* Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in monocytes infected with *S.* Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, TGN46 and Rab11a in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of TGN46 and Rab11a. Scale bar (bottom left) = 10 μm.

5.3.1.6 Localisation of VPAC1 protein with the secretory vesicular marker Rab3a in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP

Confocal study detected an involvement of TGN in the sorting and expression of VPAC1 proteins in stimulated human monocytes (Fig 5.4). Since Rab3a mediates vesicle maturation, mobilisation, and membrane fusion at synaptic junctions (Zerial and McBride, 2001), we used it as a vesicle secretion marker for VPAC1 exocytosis.

No immunoreactivity to VPAC1 or Rab3a protein was observed in controls in which monocytes were incubated with secondary antibody only (Fig 5.6A). In unstimulated monocytes cultured with both primary and secondary antibodies, weak immunoreactivity to VPAC1 and Rab3a protein was observed in the trans-membrane region of the cell but co-localisation was apparent (Fig 5.6B). When monocytes were cultured with VIP (10^{-7} M) for 24h, immunoreactivity to VPAC1 and Rab3a protein appeared to increase and co-localisation was retained (Fig 5.6C). However, when monocytes were infected with *S*. Typhimurium 4/74 for 24h increased antibody immunoreactivity to both VPAC1 and Rab3a protein was observed and these proteins were strongly co-localised within the cytoplasm and cell membrane (Fig 5.6D). When *Salmonella*-infected monocytes were co-cultured with VIP, immunoreactivity to both VPAC1 and Rab3a proteins was considerably reduced and although distribution of these proteins was altered to the transmembrane region of the cell, co-localisation was

still apparent (Fig 5.6E). In monocytes stimulated with LPS for 24h, weaker immunoreactivity to both VPAC1 and Rab3a proteins was observed when compared to *Salmonella*-infected monocytes (Fig 5.6F). In this latter case the distribution of VPAC1 and Rab3a was transmembrane but co-localisation was observed. When LPS-stimulated monocytes were co-cultured with VIP, immunoreactivity to both VPAC1 and Rab3a was considerably reduced but co-localisation to the transmembrane was retained (Fig 5.6G).

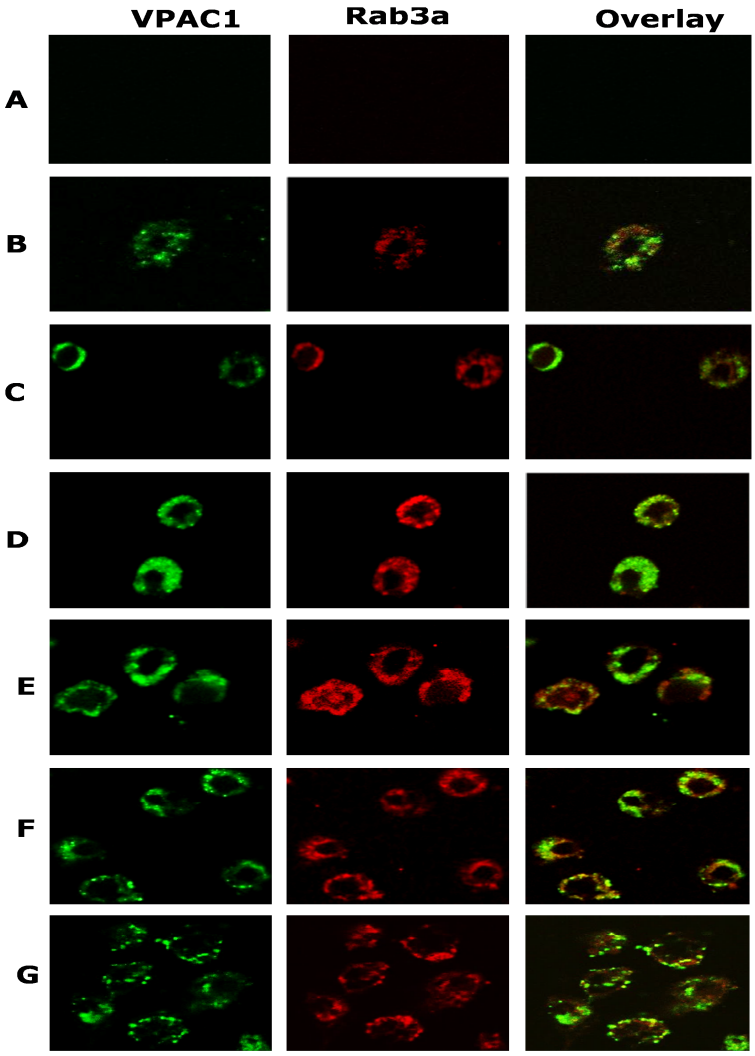

Figure 5.6: Confocal microscopy image showing the comparative immunoreactivity and distribution of VPAC1 and Rab3a protein in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to VPAC1 or Rab3a in control monocytes incubated with secondary antibodies only. Lane (B) shows the immunoreactivity to, and distribution of, VPAC1 1 and Rab3a in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, VPAC1 and Rab3a in monocytes stimulated with VIP (10^{-7} M) for 24h. Lane (D) shows the immunoreactivity to, and distribution of, VPAC1 and Rab3a in monocytes infected with *S.* Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, VPAC1 and Rab3a in monocytes infected with *S.* Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, VPAC1 and Rab3a in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, VPAC1 and Rab3a in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of VPAC1 and Rab3a. Scale bar (bottom left) = 10 μm.

5.3.1.7 Localisation of Rab3a and CaM1 protein in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP

Previous studies by Skelin and Rupnik (2011) have shown that calcium is positively correlated to cAMP activity and in turn this regulates exocytosis of several hormones and neurotransmitters (Burgoyne and Morgan, 2003). The interaction between CaM1 and Rab3a is also known to be important in the regulation of exocytosis (Yunes et al., 2002). We therefore, initially used confocal microscopy to study the localisation of CaM1 and Rab3a in human monocytes infected with *S*. Typhimurium 4/74 or stimulated with LPS and following co-culture with *Salmonella*-infected/LPS-stimulated monocytes with VIP.

No immunoreactivity to CaM1 or Rab3a protein was observed in monocytes incubated with only secondary antibodies (control cells) (Fig 5.7A). In uninfected/stimulated monocytes, only a low level of immunoreactivity associated with CaM1 protein was observed and mostly this was distributed within the transmembrane region of the cell. The distribution of Rab3a protein in these cells was also observed mostly in the transmembrane region but some was also localised to the cytoplasm, while only limited co-localisation of CaM1 and Rab3a proteins was observed (Fig 5.7B). When monocytes were cultured with VIP for 24h, the immunoreactivity and distribution/localisation of CaM1 and Rab3a protein was similar to that observed in unstimulated cells (Fig 5.7C). In contrast to these latter results, when monocytes were infected with *S*. Typhimurium 4/74 for 24h, strong immunoreactivity to

CaM1 and Rab3a protein was observed. In this latter case, CaM1 protein immunoreactivity associated was more granular in appearance and was distributed throughout the cytoplasm. The distribution of Rab3a protein was also observed throughout the cytoplasm with strong co-localisation with CaM1 (Fig 5.7D). Co-culture of *S*. Typhimurium 4/74-infected monocytes with VIP had two effects. Firstly VIP reduced the immunoreactivity associated with both CaM1 and Rab3a proteins and secondly VIP altered the distribution of these proteins from the cytoplasm (when the cells were infected with 4/74 with the addition of VIP) to the transmembrane region of the cell (Fig 5.7E). When monocytes were stimulated with LPS for 24h an intermediate level of immunoreactivity to CaM1 protein was observed (in between control and 4/74-infected cells). Transmembrane (rather than cytoplasmic) distribution of CaM1 protein was also observed in LPS-stimulated monocytes (Fig 5.7F) rather than the cytoplasmic distribution associated with *S*. Typhimurium 4/74 infection. The addition of VIP to LPS-stimulated monocytes also reduced the immunoreactivity of CaM1 and Rab3a proteins (Fig 5.7G).

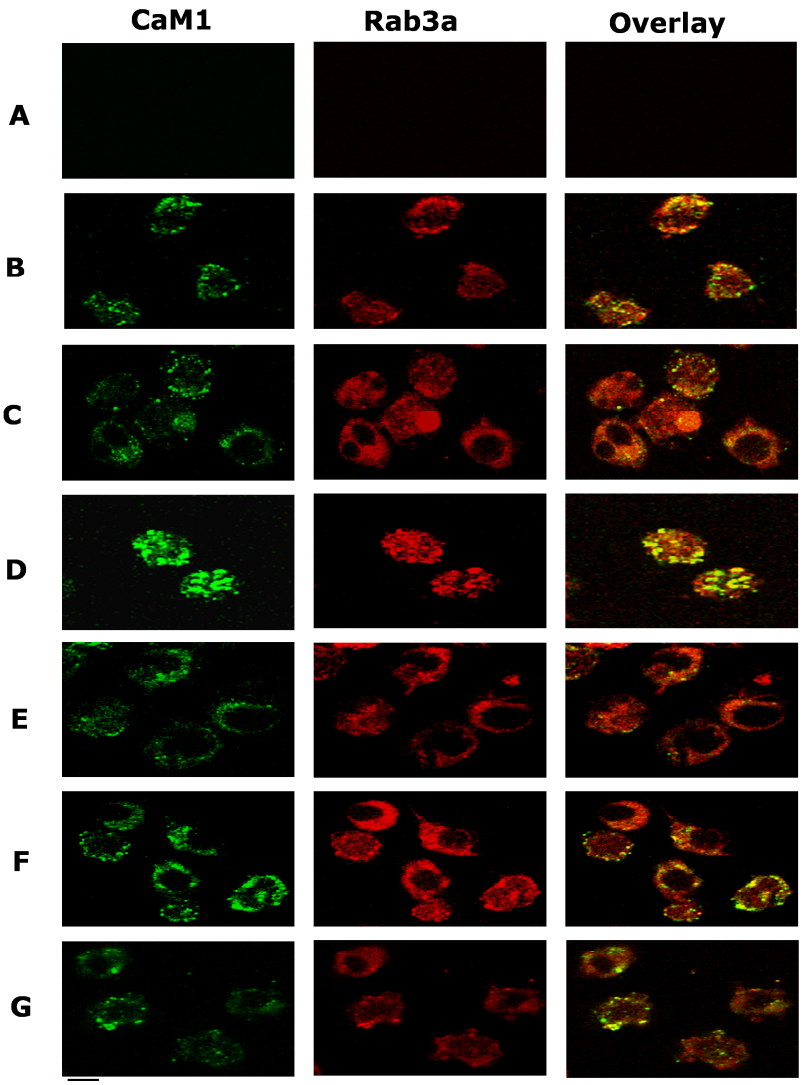

Figure 5.7: Confocal microscopy image showing the comparative immunoreactivity and distribution of CaM1 and Rab3a protein in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS and cultured with or with VIP.

Lane (A) shows no immunoreactivity to CaM1 or Rab3a protein in control monocytes incubated only with secondary antibodies. Lane (B) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in unstimulated monocytes. Lane (C) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in monocytes stimulated with VIP (10^{-7} M) for 24h. Lane (D) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in monocytes infected with *S*. Typhimurium 4/74. Lane (E) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in monocytes infected with *S*. Typhimurium 4/74 and co-cultured with VIP. Lane (F) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in monocytes stimulated with LPS (100 ng/ml) for 24h. Lane (G) shows the immunoreactivity to, and distribution of, CaM1 and Rab3a in monocytes stimulated with LPS and co-cultured with VIP. Images represent data observed from 3 separate occasions. Arrows show co-localisation of CaM1 and Rab3a. Scale bar (bottom left) = 10 μm.

5.3.2 Effect of VIP on expression of CaM1 and Rab3a mRNA in human monocytes infected with S. Typhimurium 4/74 or stimulated with LPS, with or without VIP co-culture

5.3.2.1 Effect of VIP on CaM in the stimulated monocytes

Using confocal microscopy, the results reported in this chapter suggest that immunoreactivity to both CaM1 and Rab3a protein was increased in human monocytes infected with *Salmonella* or stimulated with LPS (Fig 5.7). However, when *Salmonella*-infected or LPS-stimulated monocytes were co-cultured with VIP immunoreactivity to CaM1 and Rab3a protein appeared to decrease considerably (Fig 5.7). To investigate this effect further, the relative expression of CaM1 and Rab3a mRNA was analysed by qPCR in monocytes infected with *Salmonella* or stimulated with LPS, with or without co-culture with VIP.

Relative expression of CaM1 mRNA in unstimulated (control) monocytes was used as a reference point and given the arbitrary value of 1. When unstimulated monocytes were cultured with VIP (10^{-7} M) for 24 h, expression of CaM1 mRNA decreased but by less than 1 fold (Fig 5.8). However, expression of CaM1 mRNA significantly increased ($P < 0.01$) in monocytes infected with *S*. Typhimurium 4/74 for 24h but this decreased significantly ($P < 0.05$) in infected monocytes which were co-cultured with VIP (Fig 5.8). When monocytes were stimulated with LPS for 24h, expression of CaM1 mRNA was significantly increased ($P < 0.05$) above levels measured in unstimulated control monocytes

but significantly reduced (P <0.05) when compared to expression of CaM1 mRNA in monocytes infected with *Salmonella* (Fig 5.8). In LPS-stimulated monocytes co-cultured with VIP, expression of CaM1 mRNA was also significantly decreased (P <0.05) but remained significantly (P <0.05) above levels measured in uninfected control monocytes (Fig 5.8).

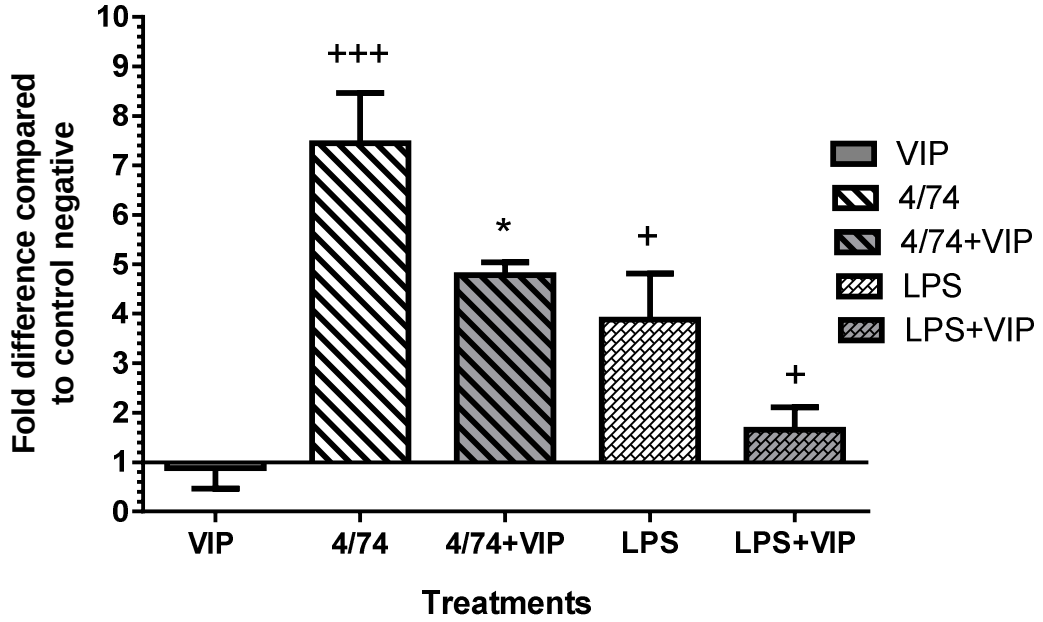

Figure 5.8: Relative expression level of CaM1 mRNA in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS with or without co-culture with VIP.

The fold change in CaM1 mRNA expression was compared to mRNA expression measured in unstimulated (control negative, C-ve) monocytes given the arbitrary expression level of 1. In treated cells, monocytes were incubated for 24h with *S*. Typhimurium 4/74 (MOI = 10) or stimulated with LPS (100 ng/ml), with or without co-culture with VIP (10^{-7} M). += Significant difference (p <0.05) and ++= very significant difference (P< 0.01) between means of LPS or bacterial-treated cells to control negative (untreated cells). While * = Significant difference (P <0.05) between means of *Salmonella* or LPS with presence or absence VIP. The experiment done on triplicates values performed on 3 separate occasions. Bars represent ±SD.

5.3.2.2 Effect of VIP on expression of Rab3a mRNA in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS, with or without VIP co-culture

Confocal microscopy analysis suggested that immunoreactivity to Rab3a protein (as well as CaM1) was increased in monocytes infected with *S*. Typhimurium 4/74 or stimulated with LPS but was reduced when these monocytes were co-cultured with VIP (Fig 5.7). To investigate this further, qPCR analysis was used and relative expression of Rab3a mRNA was compared to expression measured in unstimulated (control monocytes) in which expression was given the arbitrary value of 1. When unstimulated monocytes were cultured with VIP (10^{-7} M) for 24h, expression of Rab3a mRNA decreased but by less than 1 fold (Fig 5.9). However, expression of Rab3a mRNA significantly increased ($P < 0.05$) in monocytes infected with *S*. Typhimurium 4/74 for 24h but this decreased significantly ($P < 0.05$) in infected monocytes which were co-cultured with VIP (Fig 5.9). When monocytes were stimulated with LPS for 24h, expression of Rab3a mRNA was significantly increased ($P < 0.05$) above levels measured in unstimulated control monocytes but insignificantly reduced ($P < 0.05$) when compared to expression of CaM1 mRNA in monocytes infected with LPS co-cultured with VIP for 24h (Fig 5.9). In LPS-stimulated monocytes co-cultured with VIP, expression of Rab3a mRNA was not significantly different ($P > 0.05$) to levels measured in uninfected monocytes (Fig 5.9).

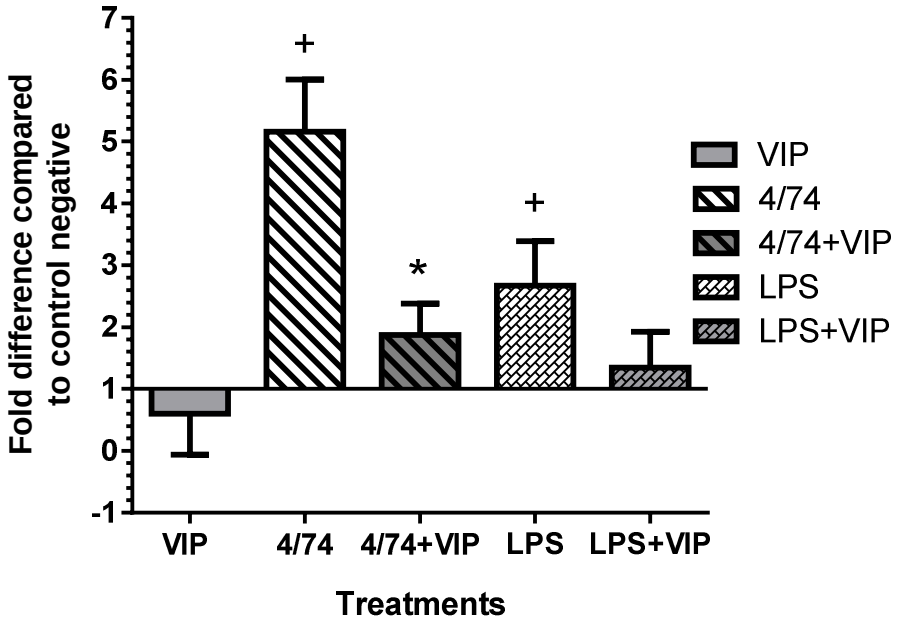

Figure 5.9: Relative expression level of Rab3a mRNA in human monocytes infected with *S.* Typhimurium 4/74 or stimulated with LPS, with or without co-culture with VIP.

The fold change in Rab3a mRNA expression was compared to mRNA expression measured in unstimulated (control negative, C-ve) monocytes given the arbitrary expression level of 1. In treated cells, monocytes were incubated for 24h with *S*. Typhimurium 4/74 (MOI = 10) or stimulated with LPS (100 ng/ml), with or without co-culture with VIP (10^{-7} M). += Significant difference (P <0.05) between means of LPS or bacterial-treated cells to control negative (untreated cells). While * = Significant difference (P <0.05) between means of *Salmonella* or LPS with presence or absence VIP. The experiment done on triplicates values performed on 3 separate occasions. Bars represent ±SD.

5.3.3 Effect of CaM on VPAC1 expression

Studies in this chapter indicated that CaM1 and Rab3a protein co-localised and that Rab3a was also co-localised to VPAC1 protein in the TGN. Furthermore, expression of both CaM1 and Rab3a mRNA were increased in *Salmonella*-infected monocytes and this up-regulation was inhibited by VIP. In chapter 4 it was shown that VPAC1 protein was increased on the surface of monocytes but this was inhibited by VIP.

Therefore, the next set of experiment was designed to investigate whether changes in CaM1 expression had a functional effect on the surface expression of VPAC1 protein.

5.3.3.1 Effect of CaM on VPAC1 mRNA expression

qPCR analysis of VPAC1 mRNA expression was performed using expression of VPAC1 mRNA in unstimulated monocytes as a reference point. Monocytes were infected with *Salmonella* or stimulated with LPS for 24h, with or without co-culture with VIP. In other experiments, CaM1 agonist (CALP1) or antagonist (W-7) was added to culture media for 2h prior to the treatments stated above.

Results showed that in uninfected monocytes, CALP1 increased VPAC1 expression by around 2 folds and W-7 decreased VPAC1 expression by about the same magnitude (Fig 5.10). The addition of VIP to monocyte cultures had very little effect on VPAC1 expression with fold changes of -0.5 (VIP only), 1.6 (VIP + CALP1) and -1.5 (VIP + W-7) (Fig 5.10). In

monocytes infected with *S*. Typhimurium 4/74, VPAC1 expression was significantly increased ($P < 0.05$) by around 6 folds when compared to uninfected monocytes, while co-culture of infected monocytes with CALP1 significantly increased ($P < 0.05$) expression of VPAC1 mRNA to >9 folds, whereas W-7 reduced the expression of VPAC1 to around 4.8 folds above that expressed by uninfected monocytes (Fig 5.10). As previously shown in this thesis, stimulation of monocytes with LPS did not increase VPAC1 expression to the same level measured in monocytes infected with *Salmonella*. In this sequence of experiments, expression of VPAC1 mRNA in LPS-stimulated monocytes increased by around 4 folds, while co-culture of monocytes with CALP1 significantly increased ($P < 0.05$) VPAC1 mRNA expression to 8 folds above that expressed by unstimulated monocytes, whereas co-culture of LPS-stimulated monocytes with W-7 reduced expression of VPAC1 mRNA to almost control levels (<2 folds increase) (Fig 5.10).

Co-culture of *Salmonella*-infected monocytes with VIP reduced VPAC1 mRNA expression (compared to *Salmonella*-infected cells not co-cultured with VIP) to about 4.5 folds above unstimulated controls (Fig 5.10). Expression of VPAC1 mRNA in *Salmonella*-infected monocytes was also reduced when the monocytes were co-cultured with VIP and the CALP1. In this case the effect of CALP1 was to increase VPAC1 mRNA expression by around 6.4 fold greater than that measured in unstimulated (control) monocytes, compared with 9.4 folds increase in the absence of VIP. The effect of the CaM antagonist W-7 was also increased by co-culture with VIP. In these monocytes expression of

VPAC1 mRNA was only increased by about 2 folds above that measured in unstimulated (control) monocytes, compared to 4.5 folds in the absence of VIP (Fig 5.10). VIP had a much lesser effect on the expression of VPAC1 mRNA in monocytes stimulated with LPS in the absence or presence of CALP1 and W-7. In LPS-stimulated monocytes co-cultured with VIP, expression of VPAC1 mRNA was increased by around 3 folds above that measured in unstimulated (control) monocytes but in LPS-stimulated monocytes, which were not co-cultured with VIP, expression of VPAC1 mRNA was only around 1 fold greater (4 folds above that measured in unstimulated monocytes). Similarly, expression of VPAC1 mRNA by LPS-stimulated monocytes co-cultured with VIP and CALP1 was about 7 folds greater than unstimulated controls but only 1 fold less than in monocytes cultured with CALP1 but not VIP. In LPS-stimulated monocytes cultured with VIP and W-7, expression of VPAC1 was only about 1 fold greater than that measured in unstimulated (control) monocytes but the level of mRNA expression in LPS-stimulated monocytes cultured with W-7 (without VIP) was only increased to about 1.5 fold above unstimulated controls (Fig 5.10).

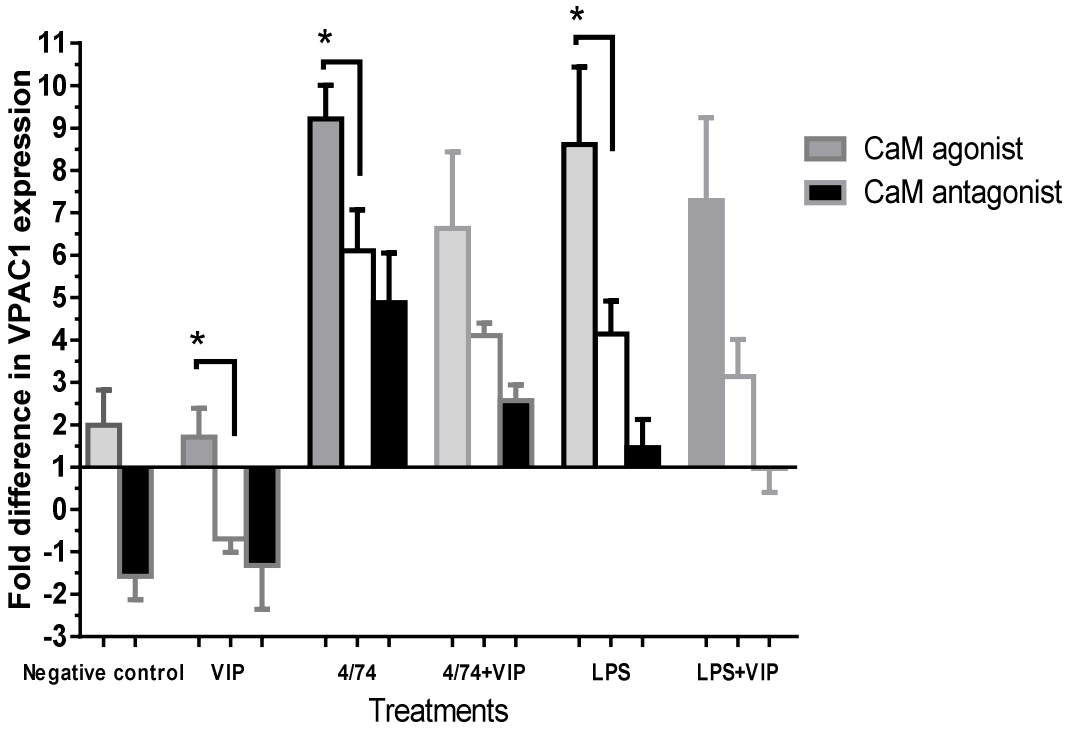

Figure 5.10: Regulatory effect of CaM on the expression level of VPAC1 receptor in human monocytes mRNA measured by qPCR

Human monocytes were incubated firstly either with CaM1 agonist (grey panels) or CaM1 antagonist (black panels) for 2h prior to culture for 24h with either VIP alone (VIP), *S*. Typhimurium 4/74, *S*. Typhimurium 4/74 + VIP, LPS or LPS+VIP. Each bar represents relative expression compared to unstimulated cells (control negative, C-ve). *=Significant difference between presence and absence of CaM agonist. The experiment done on triplicates values performed on 3 separate occasions. Deviation bars shown represent the standard deviations from the means.

5.3.3.2 Effect of CaM on VPAC1 protein expression on the cell membrane of human monocytes

In unstimulated monocytes expression of VPAC1 on the surface of monocytes was measured at 16 %, which increased to 22 % when the monocytes were cultured with the CaM agonist CALP1 and was reduced to around 8 % when the monocytes were co-cultured with the CaM antagonist W-7 (Fig 5.11A). Addition of VIP to monocyte cultures had little effect on the expression of VPAC1 with expression of 14 % (VIP only); 16.4 % when CALP1-stimulated monocytes were cultured with VIP and 9.4 % when W-7 stimulated monocytes were cultured with VIP (Fig 5.11B). When monocytes were infected with *S*. Typhimurium 4/74, about 66 % of the monocyte population expressed VPAC1 on their cell membranes. This was increased to 72 % by pre-stimulation of infected monocytes with CALP1 and reduced to about 62 % by pre-stimulation with W-7 (Fig 5.11C). In *Salmonella* infected monocytes, co-culture with VIP reduced VPAC1 expression to 39 % and although this was increased by CALP1 (to 46%). Similarly, when monocytes were stimulated with W-7 prior to infection with *Salmonella* and co-culture with VIP, VPAC1 was expressed in only 31 % of the monocyte population which was a 31 % reduction in VPAC1 expression when compared to W-7-stimulated, *Salmonella*-infected monocytes which had not been co-cultured with VIP (Fig 5.11D). Overall, LPS stimulation had much less of an effect on VPAC1 receptor expression by monocytes when compared to *Salmonella* infection. In LPS-stimulated monocytes around 28 % of the population expressed

VPAC1, compared to 66 % of monocytes infected with *Salmonella*. In monocytes pre-stimulated with CALP1, LPS increased VPAC1 expression to 34 % of the population (72 % in *Salmonella*-infected monocytes), while only 23.5 % of monocytes which had been pre-incubated with W-7 prior to LPS stimulation expressed VPAC1 (62 % in the *Salmonella*-infected monocyte population (Fig 5.11E). In LPS-stimulated monocytes, which were co-cultured with VIP, VPAC1 expression was around 22 % although this increased to about 27.2 % by CALP1 whereas decreased to 18 % in monocytes pre-stimulated with W-7 co-cultured with LPS and VIP for 24h (Fig 5.11F).

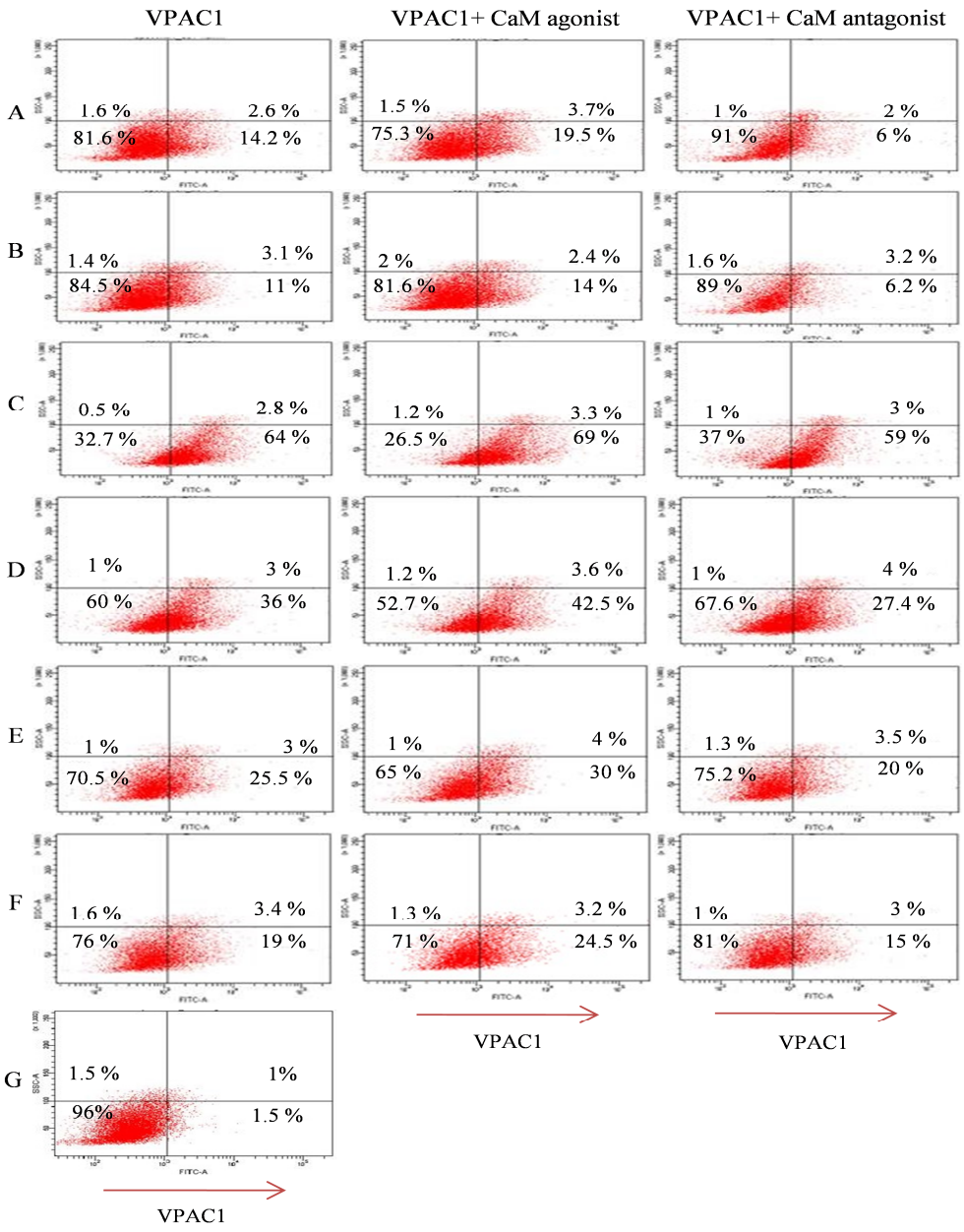

Figure 5.11: Flow cytometric analysis showing the effect of CaM on the expression of VPAC1 receptor protein by human monocytes.

Left panels show VPAC1 expression without CaM agonist/antagonist. Middle panels show VPAC1 expression following stimulation with CaM agonist (CALP1) and the right panels show VPAC1 expression following stimulation with CaM antagonist (W-7) for 2h. In Lane (A), monocytes were untreated. (B) Monocytes were co-cultured with VIP (10^{-7} M) for 24h. (C), monocytes were infected with *S*. Typhimurium 4/74 (10 MOI) for 24h. (D), monocytes were infected with *S*. Typhimurium 4/74 and co-cultured with VIP for 24h. (E), monocytes were stimulated with LPS (100 ng/ml) for 24 h. (F), monocytes were stimulated with LPS and co-cultured with VIP for 24h. (G), shows the immunoreactivity of the control (monocytes incubated only with secondary antibody). Each dot blot is representative of plots repeated on 3 separate occasions.

Results in this chapter, therefore, suggest that VPAC1 is cycled into the early endosome processed via a Rab11a positive recycling endosome prior to interaction with Rab3a and CaM1 on the TGN. Exocytosis of VPAC1 containing vesicles is, in some part, dependent on CaM1 (as shown in the schematic Fig 5.12).

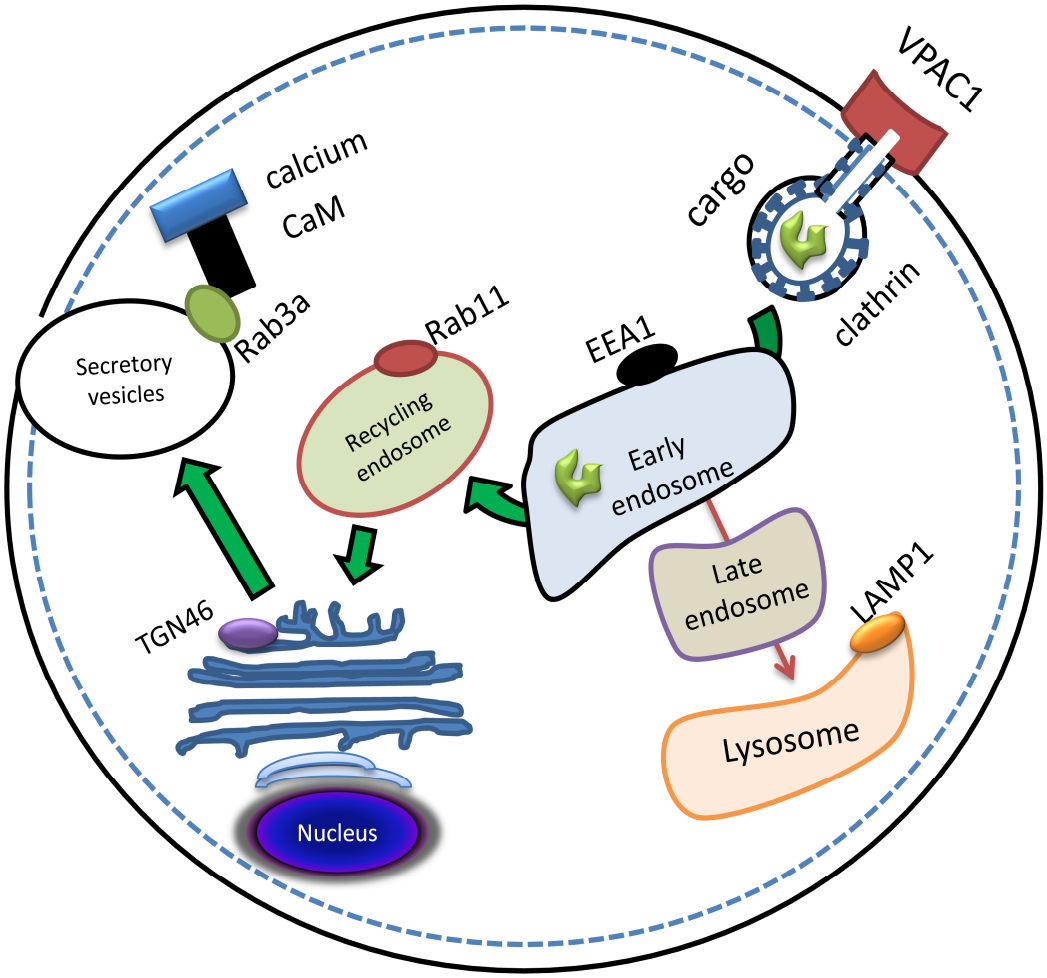

Figure 5.12: VPAC1 cycling in the human monocytes.

Green arrows denote the cycling pathway of VPAC1 in human monocytes. VPAC1 is preferentially cycled via the early endosome rather than the lysosome and is then co-localised with Rab11a in a recycling endosome. The Rab11a recycling endosome and VPAC1 then interacts with Rab3a and CaM1 on the TGN. CaM1 is then required, at least in part, for the recycling of VPAC1 to the monocyte membrane.

5.4 Discussion

VIP is a pleiotropic peptide which activates three receptors, VPAC1, VPAC2 and PAC1 (Harmar et al., 1998, Lara-Marquez et al., 2001). Data in chapter four revealed that in human monocytes, VPAC1 was the dominant of the three receptors during *Salmonella* infection or LPS stimulation. However, up-regulation of VPAC1, in response to *Salmonella* or LPS, was inhibited when the monocytes were co-cultured with VIP.

Little is known about endocytosis, degradation and/or exocytosis of VPAC1 receptor. However, some authors have reported that phosphorylation and desensitisation occur abruptly upon VIP/VPAC1 binding (McDonald et al., 1998). Like most of the GPCR family, VPAC receptors are desensitised to VIP by GRK and β-arrestin, which then induces clathrin-related proteins to internalise the phosphorylated receptor (Pierce et al., 2002). β- arrestin also prevents the internalised VPAC receptor from recycling back to the cell membrane, within 2h, in CHO (Langer et al., 2005). Furthermore, some reports have shown that VPAC1 internalisation occurs due to dynamin, another GTPase, instead of β-arrestin in human embryonic kidney (HEK) 293 cells (Claing et al., 2002, Delaney et al., 2002, Shetzline et al., 2002). Thus, the process and biochemical pathways utilised for VPAC1 endocytosis and exocytosis may differ according to species or cell type.

Experiments described in this chapter show, for the first time, some of the important molecules utilised by human monocytes for VPAC1

recycling. In unstimulated monocytes VPAC1 immunoreactivity was very low, as was the immunoreactivity of EEA1, but these did appear to be co-localised in the outer trans-membrane or sub-membrane region of the cytoplasm. When monocytes were co-cultured with VIP, only a slight increase in immunoreactivity to VPAC1 or EEA1 was observed, above unstimulated monocytes, and although co-localisation was apparent, once again this was in the trans-membrane or sub-membrane region of the cytoplasm. Studies using human HT-29 (a colo-rectal adenocarcinoma cell line) and HEK (human embryonic kidney) cells have reported that VIP rapidly induces VPAC1 internalisation (Boissard et al., 1986, Schulz et al., 2004). These results may suggest some inconsistency with the results reported in this thesis. This could be explained by cell type, since HT-29 and HEK cells are epithelial in origin, oncogene insertion or the time of exposure and concentration of VIP. In cells which co-express VPAC1 and VPAC2 receptors, VIP does not alter VPAC1 internalisation (Langer et al., 2006). However, in this latter example, Chinese hamster ovary (CHO) cells were used and so a difference in species (human versus hamster) cannot be ruled out even between this latter study and that reported in this thesis.

Immunoreactivity to both VPAC1 and EEA1 were increased in monocytes infected with *Salmonella* or stimulated with LPS and very strong co-localisation was observed. In contrast, only weak co-localisation of VPAC1 with LAMP1 was observed. This would suggest that upon endocytosis, VPAC1 is preferentially transported in the early

endosome rather than the lysosome, during infection or LPS stimulation. However, these events should not be confused with, although may be in some way linked, to numerous studies which have investigated the *Salmonella*-containing vacuole (SCV). The SCV is a modified phagosome which allows *Salmonella* to survive and replicate within the cell and requires altered regulation of many of the proteins studied in this thesis (reviewed by Steele-Mortimer, 2008). In this thesis only the association of VPAC1 with these intracellular vesicular, and their proteins, was investigated.

Increased immunoreactivity to (and very strong co-localisation between) VPAC1 and Rab11a was also detected in this study in monocytes infected with *Salmonella* or stimulated with LPS. In non-polarised cells, such as monocytes, Rab11a has a critical role in the recycling endosome due to its interaction with transferrin (Ullrich et al., 1996, Ren et al., 1998). The interaction between VPAC1 and Rab11a was also localised to the trans-Golgi-network (TGN) as measured by co-localisation of VPAC1 and Rab11a with the TGN marker, TGN46, in confocal microscopy studies. The TGN is the critical region on the Golgi apparatus which collects and sorts newly synthesised proteins and proteins transported from intracellular vesicles, such as recycling endosomes, to other regions in the cell including the cell membrane (Reviewed by Gu et al., 2001, Guo et al., 2014). Taken together, the results obtained in this study, therefore, are consistent with the hypothesis that VPAC1 is transported in the early endosome prior to a recycling step involving Rab11a and the

TGN. One study by Murray et al. (2005) has reported that LPS or IFN-γ and induces Rab11a to mediate transport of TNF-α from the TGN to the cell membrane. This is interesting since it suggests that this pathway is utilised to produce a cytokine (TNF-α) which is required in the innate immune response to LPS of Gram-negative bacteria, whereas the study reported in this thesis indicates that it is used during *Salmonella* infection, or LPS contact, to promote an immunosuppressive effect (externalisation of VPAC1).

Immunoreactivity to Rab3a (and Rab3a mRNA expression) was also investigated following *Salmonella* infection and LPS-stimulation. Rab3a is known to be associated with newly formed secretory vesicles on the TGN (Jena et al., 1994, Handley et al., 2007). Immunoreactivity to Rab3a appeared to be increased in both *Salmonella*-infected and LPS-stimulated monocytes and expression of Rab3a mRNA was significantly increased by these treatments. VPAC1 was also strongly co-localised to Rab3a and TGN46 which suggested that following interaction with the Rab11a positive recycling vesicle, VPAC1 becomes associated with a Rab3a secretory vesicle and in both cases this may be under the influence of the TGN. This may provide evidence for the pathway by which exocytosis of VPAC1 occurs, as shown by increased VPAC1 protein on the surface of monocytes infected with *Salmonella* or stimulated with LPS. Furthermore, since VIP decreased expression of Rab3a immunoreactivity, Rab3a mRNA expression and reduced co-localisation of Rab3a with VPAC1 this may explain how VIP reduces expression of VPAC1 protein on the surface of infected monocytes. In

murine bone marrow derived macrophages, LPS and inflammatory cytokines, such as IL-1, IL-6 and TNF-α, stimulate up-regulation of Rab3a (Abu-Amer et al., 1999, Zhang et al., 2013). These studies, therefore, also suggest a role for Rab3a proteins in the immune response to Gram-negative bacteria, which is beneficial to the host, rather than the immunosuppressive effect that work in this chapter (and chapter 4) suggest, which may benefit the bacteria. One puzzling aspect of this study is why co-culture with VIP inhibits *Salmonella*-induced VPAC1 expression. This would seem to be contradictory, but results shown in chapter 3 do show that VIP does inhibit the production of inflammatory mediators by infected monocytes, even though it then down-regulates the ability of the cell to be receptive to VIP.

Schlüter et al. (2002) suggested that Rab3a acts as a 'gatekeeper' in the late stages of exocytosis and interaction of Rab3a with CaM/Ca2+ is required in this process as CaM binds to arginine residues found in Rab3 but not in Rabs which are not involved in exocytosis (Regazzi, 2007). The calcium/ CaM-Rab3a complex is important since both these proteins participate in the exocytosis of many neurotransmitters and hormones (Park, 1996, Sidhu et al., 2003b). Increased CaM in murine Raw 264.7 macrophages stimulated with LPS, proceeds nuclear translocation of NF-κB and c-Jun and down-stream secretion of TNF-α and iNOS (Weber et al., 2006). Whether CaM was involved in the actual nuclear translocation or the exocytosis of pro-inflammatory mediators in this case was not studied but it is now believed that CaM

and protein kinase Ca (PKCA) are essential in exocytosis (Bernstein, 2015). Results reported in this chapter show that *Salmonella* or LPS significantly increased expression of CaM1 mRNA as well as Rab3a and that co-culture of *Salmonella*-infected or LPS-stimulated monocytes with VIP significantly inhibited this up-regulation. In *Salmonella*-infected monocytes, immunoreactivity to CaM1 was also increased and CaM1 and Rab3a were very closely co-localised, at the same time, that strong co-localisation of Rab3a with VPAC1 occurred. Since previous studies have shown the important in CaM in exocytosis (Bernstein, 2015) the next experiments were, therefore, designed to investigate whether CaM1 was important in the exocytosis of VPAC1. These results showed that a CaM agonist (CALP1) increased VPAC1 protein expression on the surface of *Salmonella*-infected monocytes, while the CaM antagonist (W-7) reduced VPAC1 expression to levels comparable with monocytes only infected with *Salmonella*. VIP inhibited expression of CaM mRNA, CaM immunoreactivity and association with Rab3a and finally the up-regulation of VPAC1 in *Salmonella*-infected monocytes cultured with CALP1. These results suggest that VPAC1 is recycled from the TGN to the cell membrane by a mechanism which requires CaM1 and possibly also Rab3a.

Chapter Six

6. General Discussion

Sepsis is a life threatening condition which etiologically occurs due to the inflammatory immune response to micro-organisms (Matot and Sprung, 2001). Sepsis has a global impact on about 18 million individuals (Ulloa and Tracey, 2005) and causes mortality rates as high as 30 % to 50 % in intensive care units (Dellinger et al., 2008). It has been demonstrated that sepsis occurs when the host immune reaction to a stimulus becomes excessive and subsequently disturbs the balance between pro and anti-inflammatory responses (Cohen, 2002). Viruses, parasites and fungi can cause sepsis (Bone et al., 1992) but Gram-negative bacteria are the most common cause of sepsis and LPS is the main bacterial component responsible for the syndrome (reviewed in Martin, 2012). Cells in the innate immune system rapidly respond to invading microorganism by producing pro-inflammatory mediators and these mediators are responsible for the induction of sepsis (Schulte et al., 2013). Monocytes are one such cell type that recognises LPS, through a CD14-TLR4 pathway, and this results in the production of numerous inflammatory mediators in blood (reviewed by Rydström and Wick, 2007).

Salmonella species are an important cause of sepsis, especially in children (Acquah et al., 2013, Randolph and McCulloh, 2014). The ability of *Salmonella* to survive inside of macrophages is a key virulence determinate (Fields et al., 1986). However, infected murine

macrophages do eventually establish effective anti-*Salmonella* killing through NADPH-induced ROS, initially, and iNOS in the later stages of infection (Mastroeni et al., 2000). *Salmonella*-infected human monocytes derived macrophages and DCs also induce considerable amounts of other antibacterial agents such as IL-12, IL-18, IFN-γ and TNF-α (Pietilä et al., 2005). Cytokine production, particularly the inflammatory cytokines, is a crucial immune response against invading microorganism (reviewed in Eckmann and Kagnoff, 2001) . However, excessive production of pro-inflammatory mediators can be detrimental, causing tissue damage and even organ collapse, as occurs in the SIRS phase of sepsis. Increased production of pro-inflammatory cytokines such as IL-6, IL-10, TNF-α and IL-18 has also been considered as biomarker of sepsis and may be monitored during treatment (Chaudhry et al., 2013). Production of IL-6 leads to the induction of acute phase proteins by the liver which, amongst other functions are involved in activation of the complement system (Kushner and Rzewnicki, 1994, Chong and Sriskandan, 2011). In plasma of septic patients, IL-6 correlates with plasma levels of IL-1β and TNF-α (Blackwell and Christman, 1996, Schulte et al., 2013) which may act as biomarkers for the severity and prognosis of sepsis (Damas et al., 1992, Burkovskiy et al., 2013). TNF-α is also an important regulator for the production of inflammatory cytokines (Parameswaran and Patial, 2010) and was regarded as the principal mediator of sepsis (Spooner et al., 1992). In the endothelial cells of septic patients, TNF-α induces expression of ICAM-1 and VCAM-1 molecules which are

required for diapedesis of immune cells from the blood to tissues (Nakae et al., 1996, Shimaoka and Park, 2008). Other studies have shown that administration of anti TNF-α antibody significantly reduced the concentration of IL-1β and IL-6 in the serum of baboons infected with *E. coli* (Fong et al., 1989) and also improved survival in septic patients (Qiu et al., 2013). Furthermore, TNF-α is regarded as a key inducer of the SIRS phase of sepsis, whilst its concentration is significantly reduced in the CARS phase of sepsis (reviewed by Jit et al., 2005). IL-1β is another important cytokine produced during many different inflammatory and auto-immune diseases (Dinarello, 2011) and is associated with septic shock (Endo et al., 1992). Administration of recombinant-human IL-1β has been associated with sepsis-like clinical signs such as haemodynamic alterations and activation of coagulation cascades in rabbits (Okusawa et al., 1988), primates (Fischer et al., 1992) and humans (Dinarello, 1997). In contrast, administration of IL-1 receptor antagonist has been shown to improve the hemodynamic response, and survival, of baboons infected with virulent *E. coli* (Fischer et al., 1992). The effect on cytokine production by immune cells, such as monocytes, is therefore an important consideration when investigating the therapeutic potential of any anti-sepsis treatment such as VIP.

Results reported in this thesis have shown that both *S.* Typhimurium infection and LPS stimulation increased the concentration of the pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and also the anti-inflammatory cytokines IL-4 and IL-10 in the supernatants of human

monocyte. Co-culture of *Salmonella*-infected, or LPS-stimulated, monocytes with VIP had no effect on the concentration of IL-4 but significantly reduced the concentration of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) whilst simultaneously increasing the concentration of IL-10 (Askar et al., 2015). Delgado et al., (1999) have shown that VIP stimulates production of IL-10 in LPS-stimulated murine macrophages via activation of the cAMP response element (CREB) and IL-10 inhibits pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, induced by LPS in human monocytes (de Waal Malefyt et al., 1991). However, it is not known whether the inhibitory effect of VIP on the production of pro-inflammatory cytokines, reported in this thesis, is due directly to the inhibitory effect of VIP on the activation of key cytokine transcriptional regulators or as an indirect result of IL-10 production. IL-10 production may also have an indirect effect on the production of pro-inflammatory cytokines. For example, Seitz et al., (1995) reported that IL-10 induced secretion of IL-Ra by human mononuclear blood cells stimulated with IL-1β. However, in this latter study it was not reported whether, or not, this led to a down-stream inhibition of the effect of IL-1β. When human monocytic THP-1 cells were stimulated with LPS for the oral pathogen *Porphyromonas gingivalis* the cells secreted both IL-18 and its most potent inhibitor, IL-18 binding protein A (IL-18BPa), but the production of IL-18BPa did not reach a high enough concentration to inhibit IL-18 (Foster et al., 2007a). However, the addition of VIP to these cultures inhibited both IL-18 and IL-18BPa and in another study, this was reported when THP-

1 cells were cultured with LPS from *E. coli* (Smalley et al., 2009). This may suggest that VIP is a 'master' inhibitor, with broad effects which override specific cytokine inhibitors.

Although the SIRS phase of sepsis is associated with elevated levels of IL-1β, TNF-α and IL-6 (Danikas et al., 2008), trials designed to inhibit these cytokines specifically (for example by using IL-1Ra) have failed. This could be due, in part, to their specific nature or the timing of therapeutic intervention (Cohen et al., 2009) and may suggest that the use of a properly timed intervention with a broad ranging immunomodulator such as VIP may have therapeutic advantage. For example, in a study by Gogos et al., (2000) it was shown that elevated serum TNF-α and IL-6 levels were associated with haemodynamic imbalance during the acute phase of sepsis and other studies have indicated that IL-6 may be used as a prognostic marker in sepsis (Hong et al., 2014) while a positive correlation has been shown between serum TNF-α concentration and the culture of Gram-negative bacteria from septic patients (Kumar and Rizvi, 2009). These studies may then suggest that the inhibitory effect of VIP on TNF-α and IL-6 production by infected, or LPS-stimulated, blood monocytes could have a therapeutic effect. However, the study by Gogos et al., (2000) also reported that an elevated IL-10/TNF-α ratio, as shown in VIP-cultured, *Salmonella*-infected or LPS-stimulated monocytes in this thesis, is correlated with a very poor prognosis in septic patients (Gogos et al., 2000). Therefore, therapeutic intervention with VIP at the wrong time could have a very detrimental effect.

Another focus of study in this thesis was to investigate the effect of VIP on the expression of cytokine receptors on the monocyte cell membrane. The results showed that even though VIP increased IL-10 production by *Salmonella*-infected monocytes, neither *Salmonella* infection or VIP altered the expression of IL-10R. Thus, *Salmonella*-infected monocytes were no more receptive to the IL-10 being produced. However, it should also be said that this model system was not studied in the presence of cytokines within the culture media which may have increased IL-10R expression but IL-6 (as well as IL-27) have been shown to increase IL-10R expression by T cells (Stumhofer et al., 2007). However, IL-6 was produced in high concentration by *Salmonella*-infected monocytes in this study and presumably had to autocrinic or paracrinic effect on IL-10R. In contrast to the result shown for IL-10R expression by *Salmonella*-infected monocytes, LPS did increase IL-10R expression and this was decreased by VIP. This may suggest that viable *Salmonella* may quorum-sense changes in the monocyte and produce proteins, for example those within the TTSS, which may suppress IL-10R expression and this cannot be altered by the concentration of VIP used in these experiments. A study by Ma et al. (2010) has shown that TNF-α modulates expression of a number of TTSS effector proteins in a variety of *S*. Typhimurium strains, although these were not actually within cells when this was measured. The effect of both *Salmonella* and VIP on the expression of TNFR.1 and IL-6R differed to that shown for IL-10R. Expression of both TNFR.1 and IL-6R proteins were increased on the surface of monocytes infected

with 4/74, or cultured with LPS for 6h. VIP had no effect on this expression by *Salmonella*-infected monocytes but did decrease expression of both receptors on the surface of monocytes cultured with LPS. Thus, VIP inhibits both the production of TNF-α and IL-6 and the ability of infected monocytes to be receptive to these proteins which could have an effect in blood monocyte populations in which cell to cell signalling via TNF-α and IL-6 may be important. For example, TNF-α signalling via TNFR.1 is associated with apoptosis in monocytes (Schneider-Brachert et al., 2004) and it has been shown that production of TNF-α by *E. coli*-infected human blood monocytes not only induces apoptosis in these cells but also induces apoptosis, via TNFR.1 ligation, in uninfected (bystander) monocytes (Dreschers et al., 2013). This latter example is perhaps very pertinent to the studies reported in this thesis since it was also shown that *S*. Typhimurium, at an MOI of 10, caused mortality in over half of the monocyte populations examined and this was significantly reduced (by around 24%) when infected monocytes were treated with VIP (Askar et al., 2015). Although TNFR.1 expression was not decreased by VIP in *Salmonella*-infected monocytes, it is possible that the reduction in TNF-α protein production, by VIP, decreased apoptosis in the monocyte populations studied. Although the percentage of monocyte mortality, following culture with *Salmonella* LPS was much less when compared to *Salmonella* infection, VIP did inhibit TNFR.1 and IL-6R on the surface of LPS-stimulated monocytes. Thus the effect of VIP on blood monocytes, and possibly the overall therapeutic potential of VIP during

sepsis, may be different depending on whether endotoxaemia or bacteraemia predominates. However, the fact that VIP reduces mortality in *Salmonella*-infected monocytes may in itself have a deleterious impact on the course of sepsis since increased apoptosis of blood monocytes has previously been correlated with survival of septic patients (Giamarellos-Bourboulis et al., 2006) and elevated monocyte numbers have also been reported to correlate with sepsis (Delano et al., 2011).

Results reported in this thesis also show that VIP increased intracellular survival of *Salmonella* in human monocytes and also in *in vivo* murine studies (the latter of which is discussed later). VIP increased survival of *Salmonell*a in human monocytes by over 2 logs during the 24h infection period. These results are consistent with results previously reported in which VIP increases survival of *S*. Typhimurium in a murine macrophage cell line (J774) by inhibiting IFN-γ-induced ROS killing pathways (Foster et al., 2005b, Foster et al., 2006). IFN-γ is essential for the up-regulation of effective ROS pathways in *Salmonella*-infected J774 cells (Foster et al., 2003) and early control of *Salmonella* infection in mice has also been shown to be IFN-γ-dependent (Kupz et al., 2014). Although the effect of *Salmonella* and/or VIP on IFN-γ pathways in human monocytes was not investigated in this thesis, these pathways are affected by *Salmonella* infection. For example, human monocytes infected with *S*. Typhimurium 14028 show increased expression of IFNG (IFN-γ gene) mRNA and this is significantly decreased by VIP, while the mRNA

expression of cytokines which inhibit IFN-γ signalling (suppressor of cytokine signalling (SOCS) are also significantly increased in *S*. Typhimurium 14028-infected monocytes which are co-cultured with VIP (Ibrahim, 2014). Furthermore, work in this previous thesis showed that interferon stimulated gene 15 (ISG15) was increased 11 fold by *Salmonella* infection but around 13 fold in *Salmonella*-infected monocytes cultured with VIP. Ubiquitin specific protease 18 (USP 18) removes ISG15 from its target proteins (Malakhova et al., 2006, Kim et al., 2006) and USP18 knockout mice are susceptible to infection with both *Salmonella* and *Mycobacterium* and subsequently to Gram-negative sepsis (Richer et al., 2010, Dauphinee et al., 2014). It is impossible to say, at this stage, whether or not a compensatory increase in USP18 occurred as a result of increased ISG15 expression due to VIP but these results suggest that it is possible that the increase in ISG15 observed may have been responsible for the increased numbers of *Salmonella* measured in monocytes after co-culture with VIP and this may also explain the results reported in chapter 3 of this thesis (and in Askar et al., 2015).

Since the entire modulatory actions of VIP occurs via VPAC receptors (VPAC1, VPAC2 and PAC1), this thesis also investigated the expression of these receptors following *S*. Typhimurium or LPS challenge and the effect of VIP on these. The results (in chapter 4) show that VPAC1 was expressed constitutively by human resting monocytes which is in accordance with previous publications (Lara-Marquez et al., 2001, El Zein et al., 2006). VPAC1 mRNA and protein expression was increased

noticeably by stimulating human monocytes with LPS and increase VPAC1 expression has been previously reported in human monocytes exposed to LPS (1 µg/ml) for 10 minutes (El Zein et al., 2006) and also in volunteers in which *E. coli* LPS (2 ng/kg body weight) was administered (Storka et al., 2013). Studies in chapter 4 also showed that VPAC1 was expressed much greater by monocytes which had been infected by *S*. Typhimurium 4/74 compared to those which had been cultured with LPS. Since, at least in mice, the suppressive effect of VIP on the production pro-inflammatory mediators is through VPAC1 (Delgado et al., 2001, Fraccaroli et al., 2009) these findings may suggest that *Salmonella* exploit the immunomodulatory action of VIP to enhance intra-cellular survival and growth. However, the addition of VIP to *Salmonella*-infected or LPS-stimulated monocytes significantly decreased expression of VPAC1 both at the mRNA and protein level. It may be possible that this represents an evolutionary counterbalance by the host cell.

Previous studies have reported that VPAC2 is not expressed by resting human monocytes (Lara-Marquez et al., 2001, El Zein et al., 2006). However, studies reported in this thesis show that VPAC2 mRNA was faintly expressed in unstimulated monocytes and that VPAC2 protein was expressed in the cytoplasm and on the surface of these monocytes. VPAC2 expression was also increased following exposure of these monocytes to *Salmonella* or LPS but to a lesser degree than VPAC1. The addition of VIP to these monocytes also had a much lesser effect in reducing VPAC2 expression compared to its effect on VPCA1.

These results are in accordance with other studies that have reported that VPAC2 expression was increased by LPS in human monocytes (Storka et al., 2013, Hauk et al., 2014) and murine macrophages (Delgado et al., 1999d, Kojima et al., 2005). However, the studies reported in this thesis are the first to show the effect of virulent *Salmonella* (rather than LPS) and VIP on the VPAC2 expression. Expression of PAC1 mRNA was also faintly detected in resting monocytes in this study, while flow cytometry also clearly showed PAC1 protein expression in the cytoplasm and cell membrane of resting monocytes. Furthermore, PAC1 expression at both the mRNA and protein level was increased by *Salmonella* infection or LPS. These results differ from El Zein et al., (2006) which reported a lack of PAC1 expression in resting monocytes and no up-regulation of PAC1 following culture with LPS. This discrepancy could be due the fact that El Zein et al., (2006) exposed the monocytes to LPS for only 10 minutes, whilst we used much longer time points (24h) and this may reflect differences in temporal expression of VIP receptors by human monocytes. The anti-inflammatory role of PAC1, through administration of PACAP, in murine model of endotoxic shock was observed by using PAC1 -/- KO mice (Martinez et al., 2002). This study indicated that PAC1 expression was crucial in inhibiting overproduction of IL-6 and nitric oxide (NO) production in mouse serum following administration of LPS. Thus, it is possible that *Salmonella* also up-regulate expression of PAC1 to reduce production of the pro-

inflammatory mediators (by inhibiting cytokines such as IL-6) which may increase survival advantage.

The results in chapter 4, therefore, have shown that of all the VIP receptors, expression of VPCA1 is most affected by *Salmonella*, LPS and VIP. The next area of study was focussed on the internalisation, degradation and recycling of VPAC1 in monocytes infected with *Salmonella*, stimulated with LPS and cultured with or without VIP (chapter 5). To date, no study has examined the internalisation and trafficking of VPAC1 in stimulated human immune cells. To do this we initially investigated whether, or not, VPAC1 was co-localised with the early endosome. Immunofluorescence microscopy indicated that *Salmonella* infection or LPS stimulation increased the fluorescence intensity of both VPAC1 and EEA1 (early endosome antigen, used as a fluorescence marker for early endosomes) in human monocytes. In both cases very strong co-localisation of VPAC1 and EEA1 was observed, suggesting that VPAC1 is internalised within the early endosome during *Salmonella* infection or when monocytes interact with LPS. Although co-culture of infected or stimulated monocytes with VIP caused a markedly reduced expression of VPAC1 protein (but not EEA1 protein) as measured by immunofluorescence, co-localisation of VPAC1 and EEA1 still remained. This may suggest that VIP inhibited the concentration of VPAC1 available on the cell membrane before being endocytosed and recycled in the early endosome. Newly formed *Salmonella*-containing vacuoles (SCVs) from the plasma membrane migrate to the early endosome (EEA1) and recruit some Rab proteins,

such as Rab4 and 5 (Steele-Mortimer et al., 1999). SCVs then recruit LAMP1 proteins during their maturation process but do not bind with lysosomes (reviewed in Harrison et al., 2004). Although *Salmonella* and LPS also increased immunofluorescence of LAMP1, and this was very noticeable reduced by co-culture with VIP, co-localisation of VPAC1 and LAMP1 was not observed. Thus, early recycling of VPAC1 from the cell membrane probably occurs via endosomes rather than lysosomes. To date, many studies have investigated the interaction of *Salmonella* with intracellular compartments. The purpose of these studies has been to understand how *Salmonella* survive and replicate in phagosomes, the role of SPI genes in this process and how *Salmonella* escape cellular lysosomes by the formation of the SCV within phagocytic cells (Mitchell et al., 2004, Madan et al., 2012). Work described in thesis has not considered interaction of *Salmonella* with these cellular compartments, merely interaction of VPAC1 with these cellular compartments in the context of *Salmonella* infection.

To further study recycling of VPAC1 from the monocyte cell membrane and cytoplasm, the role of important cytosolic proteins had to be considered. One such family of proteins studied in this thesis were the Rab proteins. Rab proteins are GTPases which are involved in membrane transport, vesicle formation and fusion (Jordens et al., 2005). They are present in cells in a GDP-associated (inactive) form or a GTP-associated (active) form, the latter of which allows their interaction with effector proteins required for membrane trafficking (reviewed by Hutagalung and Novick, 2011).

Although all Rab family proteins were not studied in this thesis, two key proteins (Rab 11a and Rab3a) were. Rab11a regulates two-way material exchange between the trans-golgi network (TGN) and the recycling endosome (Grant and Donaldson, 2009, Stenmark, 2009). To examine whether VPAC1 protein is trafficked from the endosome to the TGN via Rab11a, confocal microscopy studies were also performed. The results in chapter 5 show that VPAC1 and Rab11a are strongly co-localised and distributed within the cytoplasm of monocytes infected with *Salmonella* but in monocytes stimulated with LPS this distribution appeared to be less localised to the peri-nuclear region and more towards the cell membrane, although co-localisation was still apparent. The patterns of distribution and localisation were similar when *Salmonella*-infected or LPS-stimulated monocytes were co-cultured with VIP, although substantially less VPAC1 immunofluorescence was observed, particularly in the case of LPS and VIP cultured monocytes. Both VPAC1 and Rab11a were also co-localised to the TGN in *Salmonella*-infected or LPS-stimulated monocytes and in both cases distribution of these proteins was observed throughout the cytoplasm and in both cases immunofluorescence was reduced by co-culture of monocytes with VIP. Taken together, these results, therefore, indicate that endosomes carrying VPAC1, from the cell membrane, associate with Rab11a and are probably organised for recycling back to the cell membrane by the TGN in the endosome recycling compartment (ERC). Other studies have shown that Rab11a is involved in the transport of TNF-α to the cell membrane of murine macrophages cultured with LPS

(Murray et al., 2005) and a similar mechanism may be utilised in this example. However, internalisation of LPS from *Neisseria meningitides* by human monocyte derived dendritic cells (MDDCs) also induces co-localisation of TLR2 and TLR4 with microtubules associated with the Golgi complex but these essential receptors are not translocated to the cell membrane, which is in direct contrast to observations made in human monocytes (Uronen-Hansson et al., 2004). Thus sorting of crucial receptors by the Golgi complex may results in internal transportation, rather than transport to the cell membrane. Similarly, recruitment of TLR4 to the phagosomes of human monocytes infected with *E. coli* has been shown to be associated with Rab11a (Husebye et al., 2010). The increase in VPAC1 expression on the monocyte cell membrane, following *Salmonella* infection or LPS stimulation reported in this thesis, would suggest that association of VPAC1 with Rab11a and the TGN does induce VPAC1 recycling back to the cell membrane. However, in light of the studies discussed above, the possible role of Rab3a and CaM in intracellular transport of VPAC1 was also investigated.

Rab3a is an essential molecule in the exocytotic pathway. Nullification or over-expression of Rab3a has been shown to inhibit the final exocytosis step in PC12 cells and murine neurones (Thiagarajan et al., 2004, Schlüter et al., 2004, Schlüter et al., 2006). Martelli et al. (2000) have shown that Rab3a controls the number of secretory vesicles which are able to dock on the cell membrane and Tsuboi and

Fukuda (2006) have shown that both Rab3a and Rab27 are required in the docking process in these cells. Results in this thesis show by qPCR that expression of Rab3a mRNA is significantly increased in monocytes infected with *Salmonella* or cultured with LPS and when these cells are co-cultured with VIP, expression of Rab3a mRNA is significantly reduced. Furthermore, confocal microscopy shows that Rab3a is tightly associated with VPAC1, thus suggesting that VPAC1 is cycled to the monocyte membrane by Rab3a-dependent exocytosis. In conjunction with VPAC1 association, Rab3a was also very closely localised to CaM1. Therefore, VPAC1 associates with Rab3a/CaM1 complexes. Calmodulin binds with high affinity to Rab3 and can induce GDP to GTP conversion, thus activation, while release of Calmodulin from the Rab3/CaM complex reverses this effect (Sidhu et al., 2003b). Other results, in chapter 5, show that both *Salmonella* infection and LPS stimulation significantly increases expression of CaM1 mRNA and in both cases this is inhibited by VIP. Furthermore, when the effect of a CaM agonist and antagonist on VPAC1 was studied in chapter 5 it was shown that CaM agonist (CALP 1) increased expression of VPAC1 mRNA, while the CaM antagonist (W-7) decreased expression of VPAC1 mRNA. The effects of CALP 1 and W-7 were also reflected on the expression pattern of VPAC1 protein on the monocyte cell membrane. This data, therefore, strongly implies that VPAC1 is exported to the monocyte membrane in Rab3a/CaM exosomes.

An interesting aspect of the results discussed above is the effect of *Salmonella* on the human immune system, at least with respect to

blood monocytes, which have a very important role in sepsis. In the published data it is shown that Rab proteins are needed for transport and expression of molecules which detect Gram-negative bacteria (TLR2 and TLR4) (Uronen-Hansson et al., 2004, Husebye et al., 2010) and the production of an essential cytokine in the response to bacteria (TNF-α) (Murray et al., 2005). CaM has been reported to interact with class B GPCRs, of which VPAC1 is an example (Mahon and Shimada, 2005). While other studies have reported that increased cellular calcium and CaM are directly correlated with nuclear tranlocation of NF-κB and elevation of inflammatory cytokines in murine macrophages cultured with LPS or TNF-α (Ohmori and Hamilton, 1992, Weber et al., 2006). While calcium/calmodlin-dependent pathway have also been implicated in induction of CaMK which in turn recruits ERK, JNK, MAPK and AP-1 pathways, all of which are important in the production of the pro-inflammatory response to Gram-negative bacteria by innate immune cells (Cuschieri et al., 2005, Racioppi and Means, 2012). However, results reported in this thesis show for the first time that *Salmonella* may also utilise receptor recycling via Rab proteins, CaM and the TGN to promote greater expression of immunosuppressive receptors (VPAC1) on the monocyte membrane. This is plausible as an evolutionary mechanism to dampen the immune response and promote survival of the bacteria since the overall effect of VIP (as reported in chapter 3 and by numerous previously discussed studies) is to inhibit the pro-inflammatory pathways required to resolve infection.

However, it is rather puzzling, at this stage, why the addition of VIP to *Salmonella*-infected monocytes down-regulated this process.

References

ABAD, C., MARTINEZ, C., JUARRANZ, M. G., ARRANZ, A., LECETA, J., DELGADO, M. & GOMARIZ, R. P. 2003. Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. *Gastroenterology,* 124**,** 961-971.

ABAD, C., MARTINEZ, C., LECETA, J., JUARRANZ, M. G., DELGADO, M. & GOMARIZ, R. P. 2002. Pituitary adenylate-cyclase-activating polypeptide expression in the immune system. *Neuroimmunomodulation,* 10**,** 177-186.

ABRAHAMS, G. L. & HENSEL, M. 2006. Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells. *Cellular Microbiology,* 8**,** 728-737.

ABRAHAMS, G. L., MÜLLER, P. & HENSEL, M. 2006. Functional Dissection of SseF, a Type III Effector Protein Involved in Positioning the Salmonella-Containing Vacuole. *Traffic,* 7**,** 950-965.

ABU-AMER, Y., TEITELBAUM, S. L., CHAPPEL, J. C., SCHLESINGER, P. & ROSS, F. P. 1999. Expression and regulation of RAB3 proteins in osteoclasts and their precursors. *Journal of Bone and Mineral Research,* 14**,** 1855-1860.

ACHESON, D. & HOHMANN, E. L. 2001. Nontyphoidal salmonellosis. *Clinical Infectious Diseases,* 32**,** 263-269.

ACQUAH, S. E., QUAYE, L., SAGOE, K., ZIEM, J. B., BROMBERGER, P. I. & AMPONSEM, A. A. 2013. Susceptibility of bacterial etiological agents to commonly-used antimicrobial agents in children with sepsis at the Tamale Teaching Hospital. *BMC Infectious Diseases,* 13**,** 89.

ADAK, G. K., MEAKINS, S. M., YIP, H., LOPMAN, B. A. & O'BRIEN, S. J. 2005. Disease risks from foods, England and Wales, 1996–2000. *Emerg Infect Dis,* 11**,** 365-72.

ALEXANDER, H. R., DOHERTY, G. M., FRAKER, D. L., BLOCK, M. I., SWEDENBORG, J. E. & NORTON, J. A. 1991. Human recombinant interleukin-1α protection against the lethality of endotoxin and experimental sepsis in mice. *Journal of Surgical Research,* 50**,** 421-424.

ALTEKRUSE, S., COHEN, M. & SWERDLOW, D. 1997. Emerging foodborne diseases. *Emerging Infectious Diseases,* 3**,** 285.

ALVES-FILHO, J., DE FREITAS, A., SPILLER, F., SOUTO, F. & CUNHA, F. 2008. The role of neutrophils in severe sepsis. *Shock,* 30**,** 3.

ANDERSON, K. V., BOKLA, L. & NÜSSLEIN-VOLHARD, C. 1985. Establishment of dorsal-ventral polarity in the Drosophila

embryo: the induction of polarity by the Toll gene product. *Cell,* 42**,** 791-798.

ANDERSON, S. A., SONG, S. K., ACKERMAN, J. J. & HOTCHKISS, R. S. 1999. Sepsis increases intracellular free calcium in brain. *Journal of Neurochemistry,* 72**,** 2617-2620.

ANGUILLE, S., SMITS, E., COOLS, N., GOOSSENS, H., BERNEMAN, Z. N. & VAN TENDELOO, V. 2009. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. *J Transl Med,* 7**,** 1186.

ANGUS, D. C., LINDE-ZWIRBLE, W. T., LIDICKER, J., CLERMONT, G., CARCILLO, J. & PINSKY, M. R. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine,* 29**,** 1303-1310.

ANNANE, D., BELLISSANT, E. & CAVAILLON, J.-M. 2005. Septic shock. *The Lancet,* 365**,** 63-78.

ANTONUCCI, E., FIACCADORI, E., TACCONE, F. & VINCENT, J. L. 2014. Glucocorticoid administration in sepsis and septic shock: time for a paradigm change? *Minerva Anestesiologica,* 80**,** 1058-1062.

ARCISZEWSKI, M. B., SAND, E. & EKBLAD, E. 2008. Vasoactive intestinal peptide rescues cultured rat myenteric neurons from lipopolysaccharide induced cell death. *Regulatory Peptides,* 146**,** 218-223.

ARDA, I. S., ERGIN, F., VARAN, B., DEMIRHAN, B., ASLAN, H. & ÖZYAYLALI, I. P. 2001. Acute abdomen caused by Salmonella typhimurium infection in children. *Journal of Pediatric Surgery,* 36**,** 1849-1852.

ARRAES, S. M. A., FREITAS, M. S., DA SILVA, S. V., DE PAULA NETO, H. A., ALVES-FILHO, J. C., MARTINS, M. A., BASILE-FILHO, A., TAVARES-MURTA, B. M., BARJA-FIDALGO, C. & CUNHA, F. Q. 2006. Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation. *Blood,* 108**,** 2906-2913.

ARRANZ, A., ABAD, C., JUARRANZ, Y., TORROBA, M., ROSIGNOLI, F., LECETA, J., GOMARIZ, R. P. & MARTINEZ, C. 2006. Effect of VIP on TLR2 and TLR4 Expression in Lymph Node Immune Cells During TNBS-Induced Colitis. *Annals of the New York Academy of Sciences,* 1070**,** 129-134.

ARRANZ, A., ANDROULIDAKI, A., ZACHARIOUDAKI, V., MARTINEZ, C., MARGIORIS, A. N., GOMARIZ, R. P. & TSATSANIS, C. 2008. Vasoactive intestinal peptide suppresses toll-like receptor 4 expression in macrophages via Akt1 reducing their responsiveness to lipopolysaccharide. *Molecular Immunology,* 45**,** 2970-2980.

ASHKAR, A. A., REID, S., VERDU, E. F., ZHANG, K. & COOMBES, B. K. 2009. Interleukin-15 and NK1. 1+ cells provide innate protection

against acute Salmonella enterica serovar Typhimurium infection in the gut and in systemic tissues. *Infection and Immunity,* 77**,** 214-222.

ASKAR, B., IBRAHIM, H., BARROW, P. & FOSTER, N. 2015. Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune responses in human monocytes infected with viable Salmonella or stimulated with LPS. *Peptides,* 71**,** 188-195.

BAJAJ, V., LUCAS, R. L., HWANG, C. & LEE, C. A. 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. *Molecular Microbiology,* 22**,** 703-714.

BAKOWSKI, M. A., BRAUN, V. & BRUMELL, J. H. 2008. Salmonella-Containing Vacuoles: Directing Traffic and Nesting to Grow. *Traffic,* 9**,** 2022-2031.

BAO, S., BEAGLEY, K., FRANCE, M., SHEN, J. & HUSBAND, A. 2000. Interferon-γ plays a critical role in intestinal immunity against Salmonella typhimurium infection. *Immunology,* 99**,** 464-472.

BARCLAY, J. W., MORGAN, A. & BURGOYNE, R. D. 2005. Calcium-dependent regulation of exocytosis. *Cell Calcium,* 38**,** 343-353.

BARKSBY, H., LEA, S., PRESHAW, P. & TAYLOR, J. 2007. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clinical & Experimental Immunology,* 149**,** 217-225.

BARREIRO, L. B., MARIONI, J. C., BLEKHMAN, R., STEPHENS, M. & GILAD, Y. 2010. Functional comparison of innate immune signaling pathways in primates. *PLoS Genet,* 6**,** e1001249.

BARROW, P. A. & LOVELL, M. A. 1989. Functional homology of virulence plasmids in Salmonella gallinarum, S. pullorum, and S. typhimurium. *Infection and Immunity,* 57**,** 3136-3141.

BARYSCH, S. V., AGGARWAL, S., JAHN, R. & RIZZOLI, S. O. 2009. Sorting in early endosomes reveals connections to docking-and fusion-associated factors. *Proceedings of the National Academy of Sciences,* 106**,** 9697-9702.

BAXTER, M. A. & JONES, B. D. 2015. Two-component regulators control hilA expression by controlling fimZ and hilE expression within Salmonella enterica serovar Typhimurium. *Infection and Immunity,* 83**,** 978-985.

BEAGLEY, K. W., ELDRIDGE, J. H., KIYONO, H., EVERSON, M. P., KOOPMAN, W., HONJO, T. & MCGHEE, J. 1988. Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. *The Journal of Immunology,* 141**,** 2035-2042.

BECHTOLD, D. A., BROWN, T. M., LUCKMAN, S. M. & PIGGINS, H. D. 2008. Metabolic rhythm abnormalities in mice lacking VIP-VPAC2

signaling. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 294**,** R344-R351.

BECKER, A. J., ÜCKERT, S., STIEF, C. G., SCHELLER, F., KNAPP, W. H., MACHTENS, S. A., KUCZYK, M. A. & JONAS, U. 2002. Systemic and cavernous plasma levels of vasoactive intestinal polypeptide during sexual arousal in healthy males. *World Journal of Urology,* 20**,** 59-63.

BELLINGER, D. L., LORTON, D., BROUXHON, S., FELTEN, S. & FELTEN, D. L. 1996. The significance of vasoactive intestinal polypeptide (VIP) in immunomodulation. *Advances in Neuroimmunology,* 6**,** 5-27.

BENDER, A. T., OSTENSON, C. L., GIORDANO, D. & BEAVO, J. A. 2004. Differentiation of human monocytes in vitro with granulocyte–macrophage colony-stimulating factor and macrophage colony-stimulating factor produces distinct changes in cGMP phosphodiesterase expression. *Cellular Signalling,* 16**,** 365-374.

BERGHE, W. V., VERMEULEN, L., DE WILDE, G., DE BOSSCHER, K., BOONE, E. & HAEGEMAN, G. 2000. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochemical Pharmacology,* 60**,** 1185-1195.

BERNSTEIN, L. H. 2015. Ca2+-Stimulated Exocytosis: The Role of Calmodulin and Protein Kinase C in Ca2+ Regulation of Hormone and Neurotransmitter. *Archives of Medicine*.

BERRIDGE, M. J., BOOTMAN, M. D. & RODERICK, H. L. 2003. Calcium signalling: dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology,* 4**,** 517-529.

BHOSALE, M., KADTHUR, J. C. & NANDI, D. 2012. Roles of Salmonella enterica serovar Typhimurium encoded Peptidase N during systemic infection of Ifnγ−/− mice. *Immunobiology,* 217**,** 354-362.

BJÖRKBACKA, H., FITZGERALD, K. A., HUET, F., LI, X., GREGORY, J. A., LEE, M. A., ORDIJA, C. M., DOWLEY, N. E., GOLENBOCK, D. T. & FREEMAN, M. W. 2004. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiological Genomics,* 19**,** 319-330.

BLACKWELL, T. & CHRISTMAN, J. 1996. Sepsis and cytokines: current status. *British Journal of Anaesthesia,* 77**,** 110-117.

BOISSARD, C., MARIE, J., HEJBLUM, G., GESPACH, C. & ROSSELIN, G. 1986. Vasoactive intestinal peptide receptor regulation and reversible desensitization in human colonic carcinoma cells in culture. *Cancer Research,* 46**,** 4406-4413.

BONE, R. C., BALK, R. A., CERRA, F. B., DELLINGER, R. P., FEIN, A. M., KNAUS, W. A., SCHEIN, R. & SIBBALD, W. J. 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus

Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest Journal,* 101**,** 1644-1655.

BOUCHONNET, F., BOECHAT, N., BONAY, M. & HANCE, A. J. 2002. Alpha/beta interferon impairs the ability of human macrophages to control growth of Mycobacterium bovis BCG. *Infection and Immunity,* 70**,** 3020-3025.

BRADLEY, J. 2008. TNF-mediated inflammatory disease. *The Journal of Pathology,* 214**,** 149-160.

BRANDTZAEG, P., ØKTEDALEN, O., KIERULF, P. & OPSTAD, P. K. 1989. Elevated VIP and endotoxin plasma levels in human gram-negative septic shock. *Regulatory Peptides,* 24**,** 37-44.

BRANHAM, M. T., MAYORGA, L. S. & TOMES, C. N. 2006. Calcium-induced acrosomal exocytosis requires cAMP acting through a protein kinase A-independent, Epac-mediated pathway. *Journal of Biological Chemistry,* 281**,** 8656-8666.

BRENNAN, M. A. & COOKSON, B. T. 2000. Salmonella induces macrophage death by caspase-1-dependent necrosis. *Molecular Microbiology,* 38**,** 31-40.

BROWN, D. A., BRUCE, J. I., STRAUB, S. V. & YULE, D. I. 2004. cAMP potentiates ATP-evoked calcium signaling in human parotid acinar cells. *Journal of Biological Chemistry,* 279**,** 39485-39494.

BROZ, P. & MONACK, D. M. 2013. Noncanonical inflammasomes: caspase-11 activation and effector mechanisms. *PLoS Pathog,* 9**,** e1003144.

BRUCE, J. I., YULE, D. I. & SHUTTLEWORTH, T. J. 2002. Ca2+-dependent protein kinase-A modulation of the plasma membrane Ca2+-ATPase in parotid acinar cells. *Journal of Biological Chemistry,* 277**,** 48172-48181.

BRUNDA, M. J. 1994. Interleukin-12. *Journal of Leukocyte Biology,* 55**,** 280-288.

BUCHWALD, D. S. & BLASER, M. J. 1984. A review of human salmonellosis: II. Duration of excretion following infection with nontyphi Salmonella. *Review of Infectious Diseases,* 6**,** 345-356.

BURGOYNE, R. D. & CLAGUE, M. J. 2003. Calcium and calmodulin in membrane fusion. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1641**,** 137-143.

BURGOYNE, R. D. & MORGAN, A. 2003. Secretory granule exocytosis. *Physiological Reviews,* 83**,** 581-632.

BURKOVSKIY, I., SARDINHA, J., ZHOU, J. & LEHMANN, C. 2013. Cytokine release in sepsis. *Advances in Bioscience and Biotechnology,* 4**,** 860.

CANNON, J. G., TOMPKINS, R. G., GELFAND, J. A., MICHIE, H. R., STANFORD, G. G., VAN DER MEER, J. W., ENDRES, S., LONNEMANN, G., CORSETTI, J. & CHERNOW, B. 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *Journal of Infectious Diseases,* 161**,** 79-84.

CHANG, W.-C. & PAREKH, A. B. 2004. Close functional coupling between Ca2+ release-activated Ca2+ channels, arachidonic acid release, and leukotriene C4 secretion. *Journal of Biological Chemistry,* 279**,** 29994-29999.

CHAUDHARY, R., KARMACHARYA, S., SHRESTHA, S., DAHAL, R., MISHRA, S., BANJADE, N., RIJAL, B., SHERCHAND, J. & POKHREL, B. 2013. Incidence of Bacteremia and Septicemia in patients attending in tertiary care center, Nepal. *Journal of Institute of Medicine,* 35.

CHAUDHRY, H., ZHOU, J., ZHONG, Y., ALI, M. M., MCGUIRE, F., NAGARKATTI, P. S. & NAGARKATTI, M. 2013. Role of cytokines as a double-edged sword in sepsis. *In Vivo,* 27**,** 669-684.

CHEMINAY, C., CHAKRAVORTTY, D. & HENSEL, M. 2004. Role of neutrophils in murine salmonellosis. *Infection and Immunity,* 72**,** 468-477.

CHEN, K. W., GROß, C. J., SOTOMAYOR, F. V., STACEY, K. J., TSCHOPP, J., SWEET, M. J. & SCHRODER, K. 2014. The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. *Cell Reports,* 8**,** 570-582.

CHEN, K. W. & SCHRODER, K. 2013. Antimicrobial functions of inflammasomes. *Current Opinion in Microbiology,* 16**,** 311-318.

CHEN, L. M., KANIGA, K. & GALÁN, J. E. 1996. Salmonella spp. are cytotoxic for cultured macrophages. *Molecular Microbiology,* 21**,** 1101-1115.

CHEN, W., FENG, Y., CHEN, D. & WANDINGER-NESS, A. 1998. Rab11 Is Required for Trans-Golgi Network–to–Plasma Membrane Transport and a Preferential Target for GDP Dissociation Inhibitor. *Molecular Biology of the Cell,* 9**,** 3241-3257.

CHENG, S.-B. & FILARDO, E. J. 2012. Trans-Golgi Network (TGN) as a regulatory node for β1-adrenergic receptor (β1AR) down-modulation and recycling. *Journal of Biological Chemistry,* 287**,** 14178-14191.

CHERUBIN, C. E., NEU, H. C., IMPERATO, P. J., HARVEY, R. P. & BELLEN, N. 1974. Septicemia with non-typhoid salmonella. *Medicine,* 53**,** 365-376.

CHIA, P. Z. C. & GLEESON, P. A. 2014. Membrane tethering. *F1000prime reports,* 6.

CHICHE, L., FOREL, J.-M., THOMAS, G., FARNARIER, C., VELY, F., BLÉRY, M., PAPAZIAN, L. & VIVIER, E. 2011. The role of natural killer cells in sepsis. *BioMed Research International,* 2011.

CHIN, D. & MEANS, A. R. 2000. Calmodulin: a prototypical calcium sensor. *Trends in Cell Biology,* 10**,** 322-328.

CHOMARAT, P., BANCHEREAU, J., DAVOUST, J. & PALUCKA, A. K. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nature Immunology,* 1**,** 510-514.

CHONG, D. & SRISKANDAN, S. 2011. Pro-inflammatory mechanisms in sepsis.

CHORNY, A., GONZALEZ-REY, E. & DELGADO, M. 2006. Regulation of Dendritic Cell Differentiation by Vasoactive Intestinal Peptide. *Annals of the New York Academy of Sciences,* 1088**,** 187-194.

CHRIST-CRAIN, M. & OPAL, S. M. 2010. Clinical review: the role of biomarkers in the diagnosis and management of community-acquired pneumonia. *Crit Care,* 14**,** 203.

CLAING, A., LAPORTE, S. A., CARON, M. G. & LEFKOWITZ, R. J. 2002. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and ß-arrestin proteins. *Progress in Neurobiology,* 66**,** 61-79.

CLAPHAM, D. E. 2007. Calcium signaling. *Cell,* 131**,** 1047-1058.

CLARK, M., JEPSON, M., SIMMONS, N. & HIRST, B. 1994. Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells. *Research in Microbiology,* 145**,** 543-552.

CLARK, W. & CHRISTOPHER, K. 2000. An Introduction to DNA: Spectrophotometry, degradation, and the 'Frankengel'experiment. *Tested Studies for Laboratory Reaching,* 22**,** 81-99.

COHEN, J. 2002. The immunopathogenesis of sepsis. *Nature,* 420**,** 885-891.

COHEN, S. B., CHENG, T. T., CHINDALORE, V., DAMJANOV, N., BURGOS-VARGAS, R., DELORA, P., ZIMANY, K., TRAVERS, H. & CAULFIELD, J. P. 2009. Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis. *Arthritis & Rheumatism,* 60**,** 335-344.

COLEMAN, W., BILL, C. & BYKHOVSKAIA, M. 2007. Rab3a deletion reduces vesicle docking and transmitter release at the mouse diaphragm synapse. *Neuroscience,* 148**,** 1-6.

COLOMER, J. & MEANS, A. 2007. Physiological roles of the Ca2+/CaM-dependent protein kinase cascade in health and disease. *Calcium Signalling and Disease.* Springer.

CONTI, L., CARDONE, M., VARANO, B., PUDDU, P., BELARDELLI, F. & GESSANI, S. 2008. Role of the cytokine environment and cytokine receptor expression on the generation of functionally distinct dendritic cells from human monocytes. *European Journal of Immunology,* 38**,** 750-762.

COON, C., BEAGLEY, K. & BAO, S. 2009. The Role of Granulocyte Macrophage-Colony Stimulating Factor in Gastrointestinal Immunity to Salmonellosis. *Scandinavian Journal of Immunology,* 70**,** 106-115.

COOPER, A. M. & KHADER, S. A. 2007. IL-12p40: an inherently agonistic cytokine. *Trends in Immunology,* 28**,** 33-38.

COUPER, K. N., BLOUNT, D. G., WILSON, M. S., HAFALLA, J. C., BELKAID, Y., KAMANAKA, M., FLAVELL, R. A., DE SOUZA, J. B. & RILEY, E. M. 2008. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog,* 4**,** e1000004-e1000004.

CUA, D. J. & TATO, C. M. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nature Reviews Immunology,* 10**,** 479-489.

CUSCHIERI, J., BULGER, E., GARCIA, I., JELACIC, S. & MAIER, R. V. 2005. Calcium/calmodulin-dependent kinase II is required for platelet-activating factor priming. *Shock,* 23**,** 99-106.

CUSCHIERI, J., GOURLAY, D., BULGER, E., GARCIA, I., JELACIC, S. & MAIER, R. 2002. CALCIUM/CALMODULIN-DEPENDENT KINASE II IS REQUIRED FOR PLATELET ACTIVATING FACTOR (PAF) PRIMING OF INFLAMMATORY CELLS.: 71. *Shock,* 17**,** 24.

CUSCHIERI, J., GOURLAY, D., GARCIA, I., JELACIC, S. & MAIER, R. V. 2003. Modulation of endotoxin-induced endothelial function by calcium/calmodulin-dependent protein kinase. *Shock,* 20**,** 176-182.

DA SILVA CORREIA, J., SOLDAU, K., CHRISTEN, U., TOBIAS, P. S. & ULEVITCH, R. J. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex transfer from CD14 to TLR4 and MD-2. *Journal of Biological Chemistry,* 276**,** 21129-21135.

DAMAS, P., LEDOUX, D., NYS, M., VRINDTS, Y., DE GROOTE, D., FRANCHIMONT, P. & LAMY, M. 1992. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Annals of Surgery,* 215**,** 356.

DANIKAS, D., KARAKANTZA, M., THEODOROU, G., SAKELLAROPOULOS, G. & GOGOS, C. 2008. Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression. *Clinical & Experimental Immunology,* 154**,** 87-97.

DARVEAU, R. P., PHAM, T.-T. T., LEMLEY, K., REIFE, R. A., BAINBRIDGE, B. W., COATS, S. R., HOWALD, W. N., WAY, S. S. & HAJJAR, A. M. 2004. Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infection and Immunity,* 72**,** 5041-5051.

DARWIN, K. H. & MILLER, V. L. 1999. Molecular Basis of the Interaction ofSalmonella with the Intestinal Mucosa. *Clinical Microbiology Reviews,* 12**,** 405-428.

DAUPHINEE, S. M., RICHER, E., EVA, M. M., MCINTOSH, F., PAQUET, M., DANGOOR, D., BURKART, C., ZHANG, D.-E., GRUENHEID, S. & GROS, P. 2014. Contribution of increased ISG15, ISGylation and deregulated type I IFN signaling in Usp18 mutant mice during the course of bacterial infections. *Genes and Immunity,* 15**,** 282-292.

DAVIS, B. H. 2005. Improved diagnostic approaches to infection/sepsis detection. Expert Review of Molecular Diagnostics.

DE JONG, R., ALTARE, F., HAAGEN, I.-A., ELFERINK, D. G., DE BOER, T., VAN BREDA VRIESMAN, P. J., KABEL, P. J., DRAAISMA, J. M., VAN DISSEL, J. T. & KROON, F. P. 1998. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science,* 280**,** 1435-1438.

DE NARDO, D. 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*.

DE WAAL MALEFYT, R., ABRAMS, J., BENNETT, B., FIGDOR, C. G. & DE VRIES, J. E. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *The Journal of Experimental Medicine,* 174**,** 1209-1220.

DEIWICK, J., SALCEDO, S. P., BOUCROT, E., GILLILAND, S. M., HENRY, T., PETERMANN, N., WATERMAN, S. R., GORVEL, J.-P., HOLDEN, D. W. & MÉRESSE, S. 2006. The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. *Infection and Immunity,* 74**,** 6965-6972.

DEJAGER, L., PINHEIRO, I., BOGAERT, P., HUYS, L. & LIBERT, C. 2010. Role for neutrophils in host immune responses and genetic factors that modulate resistance to Salmonella enterica serovar typhimurium in the inbred mouse strain SPRET/Ei. *Infection and Immunity,* 78**,** 3848-3860.

DEL PRETE, G. 1998. The concept of type-1 and type-2 helper T cells and their cytokines in humans. *International Reviews of Immunology,* 16**,** 427-455.

DELANEY, K. A., MURPH, M. M., BROWN, L. M. & RADHAKRISHNA, H. 2002. Transfer of M2 muscarinic acetylcholine receptors to clathrin-derived early endosomes following clathrin-independent endocytosis. *Journal of Biological Chemistry,* 277**,** 33439-33446.

DELANO, M. J., THAYER, T., GABRILOVICH, S., KELLY-SCUMPIA, K. M., WINFIELD, R. D., SCUMPIA, P. O., CUENCA, A. G., WARNER, E., WALLET, S. M. & WALLET, M. A. 2011. Sepsis induces early alterations in innate immunity that impact mortality to secondary infection. *The Journal of Immunology,* 186**,** 195-202.

DELGADO, M., ABAD, C., MARTINEZ, C., JUARRANZ, M. G., LECETA, J., GANEA, D. & GOMARIZ, R. P. 2003. PACAP in immunity and inflammation. *Annals of the New York Academy of Sciences,* 992**,** 141-157.

DELGADO, M., ABAD, C., MARTINm, EZ, C., LECETA, J. & GOMARIZ, R. P. 2001. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. *Nature Medicine,* 7**,** 563-568.

DELGADO, M. & GANEA, D. 2001a. Inhibition of endotoxin-induced macrophage chemokine production by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide in vitro and in vivo. *The Journal of Immunology,* 167**,** 966-975.

DELGADO, M. & GANEA, D. 2001b. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit nuclear factor-κB-dependent gene activation at multiple levels in the human monocytic cell line THP-1. *Journal of Biological Chemistry,* 276**,** 369-380.

DELGADO, M. & GANEA, D. 2003. Vasoactive intestinal peptide inhibits IL-8 production in human monocytes by downregulating nuclear factor κB-dependent transcriptional activity. *Biochemical and Biophysical Research Communications,* 302**,** 275-283.

DELGADO, M., GOMARIZ, R. P., MARTINEZ, C., ABAD, C. & LECETA, J. 2000a. Anti-inflammatory properties of the type 1 and type 2 vasoactive intestinal peptide receptors: role in lethal endotoxic shock. *Shock,* 11**,** 13.

DELGADO, M., GONZALEZ-REY, E. & GANEA, D. 2005. The neuropeptide vasoactive intestinal peptide generates tolerogenic dendritic cells. *The Journal of Immunology,* 175**,** 7311-7324.

DELGADO, M., LECETA, J., SUN, W., GOMARIZ, R. P. & GANEA, D. 2000b. VIP and PACAP induce shift to a Th2 response by upregulating B7. 2 expression. *Annals of the New York Academy of Sciences,* 921**,** 68-78.

DELGADO, M., MARTINEZ, C., POZO, D., CALVO, J. R., LECETA, J., GANEA, D. & GOMARIZ, R. P. 1999a. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia

through the inhibition of TNF-α and IL-6. *The Journal of Immunology,* 162**,** 1200-1205.

DELGADO, M., MUNOZ-ELIAS, E. J., GOMARIZ, R. P. & GANEA, D. 1999b. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies. *The Journal of Immunology,* 162**,** 1707-1716.

DELGADO, M., MUNOZ-ELIAS, E. J., GOMARIZ, R. P. & GANEA, D. 1999c. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide prevent inducible nitric oxide synthase transcription in macrophages by inhibiting NF-κB and IFN regulatory factor 1 activation. *The Journal of Immunology,* 162**,** 4685-4696.

DELGADO, M., MUNOZ-ELIAS, E. J., GOMARIZ, R. P. & GANEA, D. 1999d. VIP and PACAP inhibit IL-12 production in LPS-stimulated macrophages. Subsequent effect on IFNγ synthesis by T cells. *Journal of Neuroimmunology,* 96**,** 167-181.

DELGADO, M., MUNOZ-ELIAS, E. J., KAN, Y., GOZES, I., FRIDKIN, M., BRENNEMAN, D. E., GOMARIZ, R. P. & GANEA, D. 1998. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit tumor necrosis factor α transcriptional activation by regulating nuclear factor-kB and cAMP response element-binding protein/c-jun. *Journal of Biological Chemistry,* 273**,** 31427-31436.

DELGADO, M., POZO, D. & GANEA, D. 2004. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacological Reviews,* 56**,** 249-290.

DELGADO, M., POZO, D., MARTINEZ, C., LECETA, J., CALVO, J. R., GANEA, D. & GOMARIZ, R. P. 1999e. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-α production by macrophages: in vitro and in vivo studies. *The Journal of Immunology,* 162**,** 2358-2367.

DELGADO, M., SUN, W., LECETA, J. & GANEA, D. 1999f. VIP and PACAP differentially regulate the costimulatory activity of resting and activated macrophages through the modulation of B7. 1 and B7. 2 expression. *The Journal of Immunology,* 163**,** 4213-4223.

DELLINGER, R. P., LEVY, M. M., CARLET, J. M., BION, J., PARKER, M. M., JAESCHKE, R., REINHART, K., ANGUS, D. C., BRUN-BUISSON, C. & BEALE, R. 2008. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Intensive Care Medicine,* 34**,** 17-60.

DEVI RAMNATH, R., WEING, S., HE, M., SUN, J., ZHANG, H., SINGH BAWA, M. & BHATIA, M. 2006. Inflammatory mediators in

sepsis: Cytokines, chemokines, adhesion molecules and gases. *Journal of Organ Dysfunction,* 2**,** 80-92.

DHARMANA, E., KEUTER, M., NETEA, M. G., VERSCHUEREN, I. & KULLBERG, B. J. 2001. Divergent effects of tumor necrosis factor-alpha and lymphotoxin-alpha on lethal endotoxemia and infection with live Salmonella typhimurium in mice. *European Cytokine Network,* 13**,** 104-109.

DICKSON, L., ARAMORI, I., MCCULLOCH, J., SHARKEY, J. & FINLAYSON, K. 2006. A systematic comparison of intracellular cyclic AMP and calcium signalling highlights complexities in human VPAC/PAC receptor pharmacology. *Neuropharmacology,* 51**,** 1086-1098.

DICKSON, L. & FINLAYSON, K. 2009. VPAC and PAC receptors: from ligands to function. *Pharmacology & Therapeutics,* 121**,** 294-316.

DINARELLO, C., AREND, W., SIMS, J., SMITH, D., BLUMBERG, H., O'NEILL, L., GOLDBACH-MANSKY, R., PIZARRO, T., HOFFMAN, H. & BUFLER, P. 2010. IL-1 family nomenclature. *Nature Immunology,* 11**,** 973-973.

DINARELLO, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood,* 87**,** 2095-2147.

DINARELLO, C. A. 1997. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest Journal,* 112**,** 321S-329S.

DINARELLO, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual Review of Immunology,* 27**,** 519-550.

DINARELLO, C. A. 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood,* 117**,** 3720-3732.

DONG, C. 2006. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nature Reviews Immunology,* 6**,** 329-334.

DONNELLY, R. P., DICKENSHEETS, H. & FINBLOOM, D. S. 1999. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *Journal of Interferon & Cytokine Research,* 19**,** 563-573.

DRECKTRAH, D., KNODLER, L. A., IRELAND, R. & STEELE-MORTIMER, O. 2006. The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. *Traffic,* 7**,** 39-51.

DREHER, D., KOK, M., OBREGON, C., KIAMA, S. G., GEHR, P. & NICOD, L. P. 2002. Salmonella virulence factor SipB induces activation and release of IL-18 in human dendritic cells. *Journal of Leukocyte Biology,* 72**,** 743-751.

DRESCHERS, S., GILLE, C., HAAS, M., GROSSE-OPHOFF, J., SCHNEIDER, M., LEIBER, A., BÜHRING, H.-J. & ORLIKOWSKY, T. W. 2013. Infection–induced Bystander-Apoptosis of Monocytes Is TNF-alpha-mediated.

DU, B.-H., ENG, J., HULMES, J., CHANG, M., PAN, Y.-C. & YALOW, R. 1985. Guinea pig has a unique mammalian VIP. *Biochemical and Biophysical Research Communications,* 128**,** 1093-1098.

DUMAS, J. J., MERITHEW, E., SUDHARSHAN, E., RAJAMANI, D., HAYES, S., LAWE, D., CORVERA, S. & LAMBRIGHT, D. G. 2001. Multivalent endosome targeting by homodimeric EEA1. *Molecular Cell,* 8**,** 947-958.

DUNCAN, D. J., YANG, Z., HOPKINS, P. M., STEELE, D. S. & HARRISON, S. M. 2010. TNF-α and IL-1β increase Ca 2+ leak from the sarcoplasmic reticulum and susceptibility to arrhythmia in rat ventricular myocytes. *Cell Calcium,* 47**,** 378-386.

DUNCAN, R. R., GREAVES, J., WIEGAND, U. K., MATSKEVICH, I., BODAMMER, G., APPS, D. K., SHIPSTON, M. J. & CHOW, R. H. 2003. Functional and spatial segregation of secretory vesicle pools according to vesicle age. *Nature,* 422**,** 176-180.

DVORÁKOVÁ, M. C. 2005. Cardioprotective role of the VIP signaling system. *Drug News Perspect,* 18**,** 387-391.

DYBING, J. K., WALTERS, N. & PASCUAL, D. W. 1999. Role of endogenous interleukin-18 in resolving wild-type and attenuated Salmonella typhimuriuminfections. *Infection and Immunity,* 67**,** 6242-6248.

ECHCHANNAOUI, H., FREI, K., SCHNELL, C., LEIB, S. L., ZIMMERLI, W. & LANDMANN, R. 2002. Toll-like receptor 2–deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *Journal of Infectious Diseases,* 186**,** 798-806.

ECKMANN, L., KAGNOFF, M. & FIERER, J. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infection and Immunity,* 61**,** 4569-4574.

ECKMANN, L. & KAGNOFF, M. F. 2001. Cytokines in host defense against Salmonella. *Microbes and Infection,* 3**,** 1191-1200.

EGAN, K. M., LAWSON, J. A., FRIES, S., KOLLER, B., RADER, D. J., SMYTH, E. M. & FITZGERALD, G. A. 2004. COX-2-derived prostacyclin confers atheroprotection on female mice. *Science,* 306**,** 1954-1957.

EL ZEIN, N., BADRAN, B. & SARIBAN, E. 2008. VIP differentially activates β2 integrins, CR1, and matrix metalloproteinase-9 in human monocytes through cAMP/PKA, EPAC, and PI-3K signaling pathways via VIP receptor type 1 and FPRL1. *Journal of Leukocyte Biology,* 83**,** 972-981.

EL ZEIN, N., CORAZZA, F. & SARIBAN, E. 2006. The neuropeptide pituitary adenylate cyclase activating protein is a physiological activator of human monocytes. *Cellular Signalling,* 18**,** 162-173.

EMERY, A. C. & EIDEN, L. E. 2012. Signaling through the neuropeptide GPCR PAC1 induces neuritogenesis via a single linear cAMP-and ERK-dependent pathway using a novel cAMP sensor. *The FASEB Journal,* 26**,** 3199-3211.

ENDO, S., INADA, K., INOUE, Y., KUWATA, Y., SUZUKI, M., YAMASHITA, H., HOSHI, S. & YOSHIDA, M. 1992. Two types of septic shock classified by the plasma levels of cytokines and endotoxin. *Circulatory Shock,* 38**,** 264-274.

FAHRENKRUG, J. 2010. VIP and PACAP. *Cellular Peptide Hormone Synthesis and Secretory Pathways.* Springer.

FAIX, J. D. 2013. Biomarkers of sepsis*. *Critical Reviews in Clinical Laboratory Sciences,* 50**,** 23-36.

FANTUZZI, G., ZHENG, H., FAGGIONI, R., BENIGNI, F., GHEZZI, P., SIPE, J. D., SHAW, A. R. & DINARELLO, C. A. 1996. Effect of endotoxin in IL-1 beta-deficient mice. *The Journal of Immunology,* 157**,** 291-296.

FENLON, L. A. & SLAUCH, J. M. 2014. Phagocyte Roulette in Salmonella Killing. *Cell Host & Microbe,* 15**,** 7-8.

FENTON, M. J. & GOLENBOCK, D. T. 1998. LPS-binding proteins and receptors. *Journal of Leukocyte Biology,* 64**,** 25-32.

FERRERO, J. J., ALVAREZ, A. M., RAMÍREZ-FRANCO, J., GODINO, M. C., BARTOLOMÉ-MARTÍN, D., AGUADO, C., TORRES, M., LUJÁN, R., CIRUELA, F. & SÁNCHEZ-PRIETO, J. 2013. β-Adrenergic receptors activate exchange protein directly activated by cAMP (Epac), translocate Munc13-1, and enhance the Rab3A-RIM1α interaction to potentiate glutamate release at cerebrocortical nerve terminals. *Journal of Biological Chemistry,* 288**,** 31370-31385.

FIELDS, P. I., SWANSON, R. V., HAIDARIS, C. G. & HEFFRON, F. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. *Proceedings of the National Academy of Sciences,* 83**,** 5189-5193.

FIGUEIRA, R. & HOLDEN, D. W. 2012. Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology,* 158**,** 1147-1161.

FINK, M. P. 2001. Prostaglandins and sepsis: still a fascinating topic despite almost 40 years of research. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 281**,** L534-L536.

FISCHER, E., MARANO, M. A., VAN ZEE, K. J., ROCK, C. S., HAWES, A. S., THOMPSON, W. A., DEFORGE, L., KENNEY, J., REMICK, D. &

BLOEDOW, D. 1992. Interleukin-1 receptor blockade improves survival and hemodynamic performance in Escherichia coli septic shock, but fails to alter host responses to sublethal endotoxemia. *Journal of Clinical Investigation,* 89**,** 1551.

FITZGERALD, K. A., ROWE, D. C., BARNES, B. J., CAFFREY, D. R., VISINTIN, A., LATZ, E., MONKS, B., PITHA, P. M. & GOLENBOCK, D. T. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *The Journal of Experimental Medicine,* 198**,** 1043-1055.

FLEIGE, S. & PFAFFL, M. W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine,* 27**,** 126-139.

FLUIT, A. C., JONES, M. E., SCHMITZ, F.-J., ACAR, J., GUPTA, R. & VERHOEF, J. 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. *Clinical Infectious Diseases,* 30**,** 454-460.

FOEY, A. D. & BRENNAN, F. M. 2004. Conventional protein kinase C and atypical protein kinase Cζ differentially regulate macrophage production of tumour necrosis factor-α and interleukin-10. *Immunology,* 112**,** 44-53.

FOEY, A. D., FIELD, S., AHMED, S., JAIN, A., FELDMANN, M., BRENNAN, F. M. & WILLIAMS, R. 2003. Impact of VIP and cAMP on the regulation of TNF-alpha and IL-10 production: implications for rheumatoid arthritis. *Arthritis Res Ther,* 5**,** R317-28.

FOLEY, S. & LYNNE, A. 2008. Food animal-associated challenges: Pathogenicity and antimicrobial resistance. *Journal of Animal Science,* 86**,** E173-E187.

FONG, Y., TRACEY, K. J., MOLDAWER, L., HESSE, D. G., MANOGUE, K. B., KENNEY, J., LEE, A., KUO, G., ALLISON, A. & LOWRY, S. 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. *The Journal of Experimental Medicine,* 170**,** 1627-1633.

FOREST, C. G., FERRARO, E., SABBAGH, S. C. & DAIGLE, F. 2010. Intracellular survival of Salmonella enterica serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2. *Microbiology,* 156**,** 3689-3698.

FORSBERG, M., BLOMGRAN, R., LERM, M., SÄRNDAHL, E., SEBTI, S. M., HAMILTON, A., STENDAHL, O. & ZHENG, L. 2003. Differential effects of invasion by and phagocytosis of Salmonella typhimurium on apoptosis in human macrophages: potential role of Rho–GTPases and Akt. *Journal of Leukocyte Biology,* 74**,** 620-629.

FOSTER, N. 2012. Editorial [Hot Topic: Vasoactive Intestinal Peptide (VIP): Historic Perspective and Future Potential]. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* 12**,** 303-307.

FOSTER, N., ANDREADOU, K., JAMIESON, L., PRESHAW, P. & TAYLOR, J. 2007a. VIP inhibits P. gingivalis LPS-induced IL-18 and IL-18BPa in monocytes. *Journal of Dental Research,* 86**,** 883-887.

FOSTER, N., CHEETHAM, J., TAYLOR, J. & PRESHAW, P. 2005a. VIP inhibits Porphyromonas gingivalis LPS-induced immune responses in human monocytes. *Journal of Dental Research,* 84**,** 999-1004.

FOSTER, N., HULME, S. & BARROW, P. 2003. Induction of Antimicrobial Pathways during Early-Phase Immune Response to Salmonella spp. in Murine Macrophages: Gamma Interferon (IFN-γ) and Upregulation of IFN-γ Receptor Alpha Expression Are Required for NADPH Phagocytic Oxidase gp91-Stimulated Oxidative Burst and Control of Virulent Salmonella spp. *Infection and Immunity,* 71**,** 4733-4741.

FOSTER, N., HULME, S. & BARROW, P. 2006. Vasoactive intestinal peptide (VIP) prevents killing of virulent and phoP mutant Salmonella typhimurium by inhibiting IFN-γ stimulated NADPH oxidative pathways in murine macrophages. *Cytokine,* 36**,** 134-140.

FOSTER, N., HULME, S. D. & BARROW, P. A. 2005b. Inhibition of IFN-γ-stimulated proinflammatory cytokines by vasoactive intestinal peptide (VIP) correlates with increased survival of Salmonella enterica serovar typhimurium phoP in murine macrophages. *Journal of Interferon & Cytokine Research,* 25**,** 31-42.

FOSTER, N., LEA, S. R., PRESHAW, P. M. & TAYLOR, J. J. 2007b. Pivotal advance: vasoactive intestinal peptide inhibits up-regulation of human monocyte TLR2 and TLR4 by LPS and differentiation of monocytes to macrophages. *J Leukoc Biol,* 81**,** 893-903.

FRACCAROLI, L., ALFIERI, J., LAROCCA, L., CALAFAT, M., ROCA, V., LOMBARDI, E., RAMHORST, R. & LEIRÓS, C. P. 2009. VIP modulates the pro-inflammatory maternal response, inducing tolerance to trophoblast cells. *British Journal of Pharmacology,* 156**,** 116-126.

FUSAKIO, M. E., MOHAMMED, J. P., LAUMONNIER, Y., HOEBE, K., KÖHL, J. & MATTNER, J. 2011. C5a regulates NKT and NK cell functions in sepsis. *The Journal of Immunology,* 187**,** 5805-5812.

GABBAY-BENZIV, R., AO, A., FISCH, B., ZHANG, L., ORON, G., KESSLER-ICEKSON, G., BEN-HAROUSH, A., KRISSI, H. & ABIR,

R. 2012. Vasoactive intestinal peptide and its receptors in human ovarian cortical follicles. *PloS One*:7, e37015.

GAFFEN, S. L., HERNÁNDEZ-SANTOS, N. & PETERSON, A. C. 2011. IL-17 signaling in host defense against Candida albicans. *Immunologic Research,* 50**,** 181-187.

GALÁN, J. E. 1999. Interaction of Salmonella with host cells through the centisome 63 type III secretion system. *Current Opinion in Microbiology,* 2**,** 46-50.

GALLUZZI, L. & KROEMER, G. 2008. Necroptosis: a specialized pathway of programmed necrosis. *Cell,* 135**,** 1161-1163.

GANDHAPUDI, S. K., TAN, C., MARINO, J. H., TAYLOR, A. A., PACK, C. C., GAIKWAD, J., VAN DE WIELE, C. J., WREN, J. D. & TEAGUE, T. K. 2015. IL-18 Acts in Synergy with IL-7 To Promote Ex Vivo Expansion of T Lymphoid Progenitor Cells. *The Journal of Immunology,* 194**,** 3820-3828.

GANEA, D. & DELGADO, M. 2002. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. *Critical Reviews in Oral Biology & Medicine,* 13**,** 229-237.

GARRETT, M. D., KABCENELL, A. K., ZAHNER, J. E., KAIBUCHI, K., SASAKI, T., TAKAI, Y., CHENEY, C. M. & NOVICK, P. J. 1993. Interaction of Sec4 with GDI proteins from bovine brain, Drosophila melanogaster and Saccharomyces cerevisiae: Conservation of GDI membrane dissociation activity. *FEBS letters,* 331**,** 233-238.

GHAFERI, A. A., CHOJNACKI, K. A., LONG, W. D., CAMERON, J. L. & YEO, C. J. 2008. Pancreatic VIPomas: subject review and one institutional experience. *Journal of Gastrointestinal Surgery,* 12**,** 382-393.

GIAMARELLOS-BOURBOULIS, E. J., ROUTSI, C., PLACHOURAS, D., MARKAKI, V., RAFTOGIANNIS, M., ZERVAKIS, D., KOUSSOULAS, V., ORFANOS, S., KOTANIDOU, A. & ARMAGANIDIS, A. 2006. Early apoptosis of blood monocytes in the septic host: is it a mechanism of protection in the event of septic shock? *Critical Care,* 10**,** R76.

GIAMARELLOS-BOURBOULIS, E. J., VAN DE VEERDONK, F. L., MOUKTAROUDI, M., RAFTOGIANNIS, M., ANTONOPOULOU, A., JOOSTEN, L., PICKKERS, P., SAVVA, A., GEORGITSI, M. & VAN DER MEER, J. 2011. Inhibition of caspase-1 activation in Gram-negative sepsis and experimental endotoxemia. *Crit Care,* 15**,** R27.

GILLESSEN, S., CARVAJAL, D., LING, P., PODLASKI, F. J., STREMLO, D. L., FAMILLETTI, P. C., GUBLER, U., PRESKY, D. H., STERN, A. S. & GATELY, M. K. 1995. Mouse interleukin-12 (IL-12) p40

homodimer: a potent IL-12 antagonist. *European Journal of Immunology,* 25**,** 200-206.

GLYNN, M. K., BOPP, C., DEWITT, W., DABNEY, P., MOKHTAR, M. & ANGULO, F. J. 1998. Emergence of Multidrug-Resistant Salmonella enterica SerotypeTyphimurium DT104 Infections in the United States. *New England Journal of Medicine,* 338**,** 1333-1339.

GODINEZ, I., KEESTRA, A. M., SPEES, A. & BÄUMLER, A. J. 2011. The IL-23 axis in Salmonella gastroenteritis. *Cellular Microbiology,* 13**,** 1639-1647.

GOETZL, E. J., VOICE, J. K., SHEN, S., DORSAM, G., KONG, Y., WEST, K. M., MORRISON, C. F. & HARMAR, A. J. 2001. Enhanced delayed-type hypersensitivity and diminished immediate-type hypersensitivity in mice lacking the inducible VPAC2 receptor for vasoactive intestinal peptide. *Proceedings of the National Academy of Sciences,* 98**,** 13854-13859.

GOGOS, C. A., DROSOU, E., BASSARIS, H. P. & SKOUTELIS, A. 2000. Pro-versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *Journal of Infectious Diseases,* 181**,** 176-180.

GOMARIZ, R. P., ARRANZ, A., ABAD, C., TORROBA, M., MARTINEZ, C., ROSIGNOLI, F., GARCIA-GÓMEZ, M., LECETA, J. & JUARRANZ, Y. 2005. Time-course expression of Toll-like receptors 2 and 4 in inflammatory bowel disease and homeostatic effect of VIP. *Journal of Leukocyte Biology,* 78**,** 491-502.

GONG, J., WEI, T., ZHANG, N., JAMITZKY, F., HECKL, W. M., RÖSSLE, S. C. & STARK, R. W. 2010. TollML: a database of toll-like receptor structural motifs. *Journal of Molecular Modeling,* 16**,** 1283-1289.

GONZALEZ-REY, E., CHORNY, A. & DELGADO, M. 2007a. Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nature Reviews Immunology,* 7**,** 52-63.

GONZALEZ-REY, E., VARELA, N., CHORNY, A. & DELGADO, M. 2007b. Therapeutical approaches of vasoactive intestinal peptide as a pleiotropic immunomodulator. *Current Pharmaceutical Design,* 13**,** 1113-1139.

GONZALEZ–REY, E. & DELGADO, M. 2006. Therapeutic treatment of experimental colitis with regulatory dendritic cells generated with vasoactive intestinal peptide. *Gastroenterology,* 131**,** 1799-1811.

GORDON, M. A. 2011. Invasive Non-typhoidal Salmonella Disease–epidemiology, pathogenesis and diagnosis. *Current Opinion in Infectious Diseases,* 24**,** 484.

GORDON, M. A., JACK, D. L., DOCKRELL, D. H., LEE, M. E. & READ, R. C. 2005. Gamma interferon enhances internalization and early

nonoxidative killing of Salmonella enterica serovar Typhimurium by human macrophages and modifies cytokine responses. *Infection and Immunity,* 73**,** 3445-3452.

GORDON, M. A., KANKWATIRA, A. M., MWAFULIRWA, G., WALSH, A. L., HOPKINS, M. J., PARRY, C. M., FARAGHER, E. B., ZIJLSTRA, E. E., HEYDERMAN, R. S. & MOLYNEUX, M. E. 2010. Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. *Clinical Infectious Diseases,* 50**,** 953-962.

GOZES, I., PERL, O., GILADI, E., DAVIDSON, A., ASHUR-FABIAN, O., RUBINRAUT, S. & FRIDKIN, M. 1999. Mapping the active site in vasoactive intestinal peptide to a core of four amino acids: neuroprotective drug design. *Proceedings of the National Academy of Sciences,* 96**,** 4143-4148.

GRANDEL, U., FINK, L., BLUM, A., HEEP, M., BUERKE, M., KRAEMER, H.-J., MAYER, K., BOHLE, R. M., SEEGER, W. & GRIMMINGER, F. 2000. Endotoxin-Induced Myocardial Tumor Necrosis Factor-α Synthesis Depresses Contractility of Isolated Rat Hearts Evidence for a Role of Sphingosine and Cyclooxygenase-2–Derived Thromboxane Production. *Circulation,* 102**,** 2758-2764.

GRANT, B. D. & DONALDSON, J. G. 2009. Pathways and mechanisms of endocytic recycling. *Nature Reviews Molecular Cell Biology,* 10**,** 597-608.

GRASSO, E., PAPARINI, D., HAUK, V., SALAMONE, G., LEIROS, C. P. & RAMHORST, R. 2014. Differential migration and activation profile of monocytes after trophoblast interaction. *PloS One,* 9**,** e97147.

GRONEBERG, D. A., HARTMANN, P., DINH, Q. T. & FISCHER, A. 2001. Expression and distribution of vasoactive intestinal polypeptide receptor VPAC2 mRNA in human airways. *Laboratory Investigation,* 81**,** 749-755.

GU, F., CRUMP, C. & THOMAS, G. 2001. Trans-Golgi network sorting. *Cellular and Molecular Life Sciences CMLS,* 58**,** 1067-1084.

GUINEY, D. 2005. The role of host cell death in Salmonella infections. *Role of Apoptosis in Infection.* Springer.

GULLO, A., ISCRA, F., DI CAPUA, G., BERLOT, G., LUCANGELO, U., CHIEREGO, M., RISTAGNO, G., PERATONER, A., FASIOLO, S. & CONSALES, C. 2005. Sepsis and organ dysfunction: an ongoing challenge. *Minerva Anestesiologica,* 71**,** 671-699.

GUO, Y., SIRKIS, D. W. & SCHEKMAN, R. 2014. Protein sorting at the trans-Golgi Network. *Annual Review of Cell and Developmental Biology,* 30**,** 169-206.

GUTIERREZ-CANAS, I., JUARRANZ, Y., SANTIAGO, B., ARRANZ, A., MARTINEZ, C., GALINDO, M., PAYA, M., GOMARIZ, R. & PABLOS, J. 2006. VIP down-regulates TLR4 expression and

TLR4-mediated chemokine production in human rheumatoid synovial fibroblasts. *Rheumatology,* 45**,** 527-532.

HACK, C. E., DE GROOT, E. R., FELT-BERSMA, R., NUIJENS, J. H., VAN SCHIJNDEL, R. S., EERENBERG-BELMER, A., THIJS, L. G. & AARDEN, L. A. 1989. Increased plasma levels of interleukin-6 in sepsis. *Blood,* 74**,** 1704-1710.

HAILMAN, E., LICHENSTEIN, H. S., WURFEL, M. M., MILLER, D. S., JOHNSON, D. A., KELLEY, M., BUSSE, L. A., ZUKOWSKI, M. M. & WRIGHT, S. D. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *The Journal of Experimental Medicine,* 179**,** 269-277.

HAMELINK, C., LEE, H.-W., CHEN, Y., GRIMALDI, M. & EIDEN, L. E. 2002. Coincident elevation of cAMP and calcium influx by PACAP-27 synergistically regulates vasoactive intestinal polypeptide gene transcription through a novel PKA-independent signaling pathway. *The Journal of Neuroscience,* 22**,** 5310-5320.

HAMZA, T., BARNETT, J. B. & LI, B. 2010. Interleukin 12 a key immunoregulatory cytokine in infection applications. *International Journal of Molecular Sciences,* 11**,** 789-806.

HANDLEY, M. T., HAYNES, L. P. & BURGOYNE, R. D. 2007. Differential dynamics of Rab3A and Rab27A on secretory granules. *Journal of Cell Science,* 120**,** 973-984.

HARFI, I., CORAZZA, F., D'HONDT, S. & SARIBAN, E. 2005. Differential calcium regulation of proinflammatory activities in human neutrophils exposed to the neuropeptide pituitary adenylate cyclase-activating protein. *The Journal of Immunology,* 175**,** 4091-4102.

HARMAR, A. J., ARIMURA, A., GOZES, I., JOURNOT, L., LABURTHE, M., PISEGNA, J. R., RAWLINGS, S. R., ROBBERECHT, P., SAID, S. I. & SREEDHARAN, S. P. 1998. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacological Reviews,* 50**,** 265-270.

HARMAR, A. J., SHEWARD, W. J., MORRISON, C. F., WASER, B., GUGGER, M. & REUBI, J. C. 2004. Distribution of the VPAC2 receptor in peripheral tissues of the mouse. *Endocrinology,* 145**,** 1203-1210.

HARRIS, K. M. 2011. Monocytes differentiated with GM-CSF and IL-15 initiate Th17 and Th1 responses that are contact-dependent and mediated by IL-15. *Journal of Leukocyte Biology,* 90**,** 727-734.

HARRIS, K. M., FASANO, A. & MANN, D. L. 2010. Monocytes differentiated with IL-15 support Th17 and Th1 responses to wheat gliadin: implications for celiac disease. *Clinical Immunology,* 135**,** 430-439.

HARRISON, D. A., WELCH, C. A. & EDDLESTON, J. M. 2006. The epidemiology of severe sepsis in England, Wales and Northern Ireland, 1996 to 2004: secondary analysis of a high quality clinical database, the ICNARC Case Mix Programme Database. *Crit Care,* 10**,** R42.

HARRISON, R. E., BRUMELL, J. H., KHANDANI, A., BUCCI, C., SCOTT, C. C., JIANG, X., FINLAY, B. B. & GRINSTEIN, S. 2004. Salmonella impairs RILP recruitment to Rab7 during maturation of invasion vacuoles. *Molecular Biology of the Cell,* 15**,** 3146-3154.

HATAKEYAMA, H., TAKAHASHI, N., KISHIMOTO, T., NEMOTO, T. & KASAI, H. 2007. Two cAMP-dependent pathways differentially regulate exocytosis of large dense-core and small vesicles in mouse β-cells. *The Journal of Physiology,* 582**,** 1087-1098.

HAUK, V., FRACCAROLI, L., GRASSO, E., EIMON, A., RAMHORST, R., HUBSCHER, O. & PEREZ LEIROS, C. 2014. Monocytes from Sjögren's syndrome patients display increased vasoactive intestinal peptide receptor 2 expression and impaired apoptotic cell phagocytosis. *Clinical & Experimental Immunology,* 177**,** 662-670.

HAYWARD, M. R., JANSEN, V. A. & WOODWARD, M. J. 2013. Comparative genomics of Salmonella enterica serovars Derby and Mbandaka, two prevalent serovars associated with different livestock species in the UK. *BMC Genomics,* 14**,** 365.

HENNING, R. J. & SAWMILLER, D. R. 2001. Vasoactive intestinal peptide: cardiovascular effects. *Cardiovascular Research,* 49**,** 27-37.

HENSEL, M. 2000. Salmonella pathogenicity island 2. *Molecular Microbiology,* 36**,** 1015-1023.

HERRERA, J. L., GONZALEZ-REY, E., FERNANDEZ-MONTESINOS, R., QUINTANA, F. J., NAJMANOVICH, R. & POZO, D. 2009. Toll-like receptor stimulation differentially regulates vasoactive intestinal peptide type 2 receptor in macrophages. *Journal of Cellular and Molecular Medicine,* 13**,** 3209-3217.

HESSE, D., TRACEY, K., FONG, Y., MANOGUE, K., PALLADINO JR, M., CERAMI, A., SHIRES, G. & LOWRY, S. 1988. Cytokine appearance in human endotoxemia and primate bacteremia. *Surgery, Oynecology & Obstetrics,* 166**,** 147-153.

HIGGINS, J., MUTAMBA, S., MAHIDA, Y., BARROW, P. & FOSTER, N. 2015. IL-36α induces maturation of Th1-inducing human MDDC and synergises with IFN-γ to induce high surface expression of CD14 and CD11c. *Human Immunology,* 76**,** 245-253.

HIJDRA, D., VORSELAARS, A. D., GRUTTERS, J. C., CLAESSEN, A. M. & RIJKERS, G. T. 2012. Differential expression of TNFR1 (CD120a)

and TNFR2 (CD120b) on subpopulations of human monocytes. *J Inflamm (Lond),* 9**,** 38.

HONG, T.-H., CHANG, C.-H., KO, W.-J., LIN, C.-F., LIU, H.-H., CHOW, L.-P., HUANG, C.-T., YU, S.-L. & CHEN, Y.-S. 2014. Biomarkers of early sepsis may be correlated with outcome. *J Transl Med,* 26**,** 1.

HOPKINS, C., MILLER, K. & BEARDMORE, J. 1985. Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. *Journal of Cell Science,* 1985**,** 173-186.

HORIUCHI, T., MITOMA, H., HARASHIMA, S.-I., TSUKAMOTO, H. & SHIMODA, T. 2010. Transmembrane TNF-α: structure, function and interaction with anti-TNF agents. *Rheumatology,* 49**,** 1215-1228.

HOTCHKISS, R. S. & KARL, I. E. 2003. The pathophysiology and treatment of sepsis. *New England Journal of Medicine,* 348**,** 138-150.

HUGHES, A. T., GUILDING, C. & PIGGINS, H. D. 2011. Neuropeptide signaling differentially affects phase maintenance and rhythm generation in SCN and extra-SCN circadian oscillators. *PloS one,* 6**,** e18926.

HUMPHREYS, D., HUME, P. J. & KORONAKIS, V. 2009. The Salmonella effector SptP dephosphorylates host AAA+ ATPase VCP to promote development of its intracellular replicative niche. *Cell Host & Microbe,* 5**,** 225-233.

HUNTER, C. A., ELLIS-NEYES, L. A., SLIFER, T., KANALY, S., GRÜNIG, G., FORT, M., RENNICK, D. & ARAUJO, F. G. 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *The Journal of Immunology,* 158**,** 3311-3316.

HUOTARI, J. & HELENIUS, A. 2011. Endosome maturation. *The EMBO Journal,* 30**,** 3481-3500.

HUSEBYE, H., AUNE, M. H., STENVIK, J., SAMSTAD, E., SKJELDAL, F., HALAAS, Ø., NILSEN, N. J., STENMARK, H., LATZ, E. & LIEN, E. 2010. The Rab11a GTPase controls Toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes. *Immunity,* 33**,** 583-596.

HUTAGALUNG, A. H. & NOVICK, P. J. 2011. Role of Rab GTPases in membrane traffic and cell physiology. *Physiological Reviews,* 91**,** 119-149.

HYDUK, S. J., CHAN, J. R., DUFFY, S. T., CHEN, M., PETERSON, M. D., WADDELL, T. K., DIGBY, G. C., SZASZI, K., KAPUS, A. & CYBULSKY, M. I. 2007. Phospholipase C, calcium, and calmodulin are critical for α4β1 integrin affinity up-regulation

and monocyte arrest triggered by chemoattractants. *Blood,* 109**,** 176-184.

IBRAHIM, H., BARROW, P. & FOSTER, N. 2011. Transcriptional modulation by VIP: a rational target against inflammatory disease. *Clinical Epigenetics,* 2**,** 213-222.

IBRAHIM, H. K. 2014. *The therapeutic potential of vasoactive intestinal peptide in human Gram-negative induced sepsis.* PhD PhD Thesis, Nottingham.

IGARASHI, H., FUJIMORI, N., ITO, T., NAKAMURA, T., OONO, T., NAKAMURA, K., SUZUKI, K., JENSEN, R. T. & TAKAYANAGI, R. 2011. Vasoactive Intestinal Peptide (VIP) and VIP Receptors-Elucidation of Structure and Function for Therapeutic Applications.

INAGAKI, N., YOSHIDA, H., MIZUTA, M., MIZUNO, N., FUJII, Y., GONOI, T., MIYAZAKI, J.-I. & SEINO, S. 1994. Cloning and functional characterization of a third pituitary adenylate cyclase-activating polypeptide receptor subtype expressed in insulin-secreting cells. *Proceedings of the National Academy of Sciences,* 91**,** 2679-2683.

ISHIHARA, T., SHIGEMOTO, R., MORI, K., TAKAHASHI, K. & NAGATA, S. 1992. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron,* 8**,** 811-819.

IVANEK, R., SNARY, E. L. & COOK, A. J. 2004. A mathematical model for the transmission of Salmonella Typhimurium within a grower-finisher pig herd in Great Britain. *Journal of Food Protection®,* 67**,** 2403-2409.

IWAKURA, Y., ISHIGAME, H., SAIJO, S. & NAKAE, S. 2011. Functional specialization of interleukin-17 family members. *Immunity,* 34**,** 149-162.

JACOBI, J. 2002. Sepsis: A Frequent, Life-Threatening Syndrome. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy,* 22**,** 169S-181S.

JANEWAY, C. A., TRAVERS, P., WALPORT, M. & SHLOMCHIK, M. J. 2001. *Basic concepts in Immunology*.

JANG, Y., LEE, S., LIM, J., LEE, H., KIM, T., PARK, J., CHUNG, B. & CHOE, N. 2008. The rate of Salmonella spp. infection in zoo animals at Seoul Grand Park, Korea. *Journal of Veterinary Science,* 9**,** 177-181.

JENA, B. P., GUMKOWSKI, F. D., KONIECZKO, E. M., VON MOLLARD, G. F., JAHN, R. & JAMIESON, J. D. 1994. Redistribution of a rab3-like GTP-binding protein from secretory granules to the Golgi complex in pancreatic acinar cells during regulated exocytosis. *The Journal of cell biology,* 124**,** 43-53.

JENKINS, M. K., KHORUTS, A., INGULLI, E., MUELLER, D. L., MCSORLEY, S. J., REINHARDT, R. L., ITANO, A. & PAPE, K. A. 2001. In vivo activation of antigen-specific CD4 T cells. *Annual Review of Immunology,* 19**,** 23-45.

JEPSON, M. A. & CLARK, M. A. 2001. The role of M cells in Salmonella infection. *Microbes and Infection,* 3**,** 1183-1190.

JIT, M., HENDERSON, B., STEVENS, M. & SEYMOUR, R. 2005. TNF-α neutralization in cytokine-driven diseases: a mathematical model to account for therapeutic success in rheumatoid arthritis but therapeutic failure in systemic inflammatory response syndrome. *Rheumatology,* 44**,** 323-331.

JONES, P., COLLINS, P. & AITKEN, M. 1988. Passive protection of calves against experimental infection with Salmonella typhimurium. *The Veterinary Record,* 123**,** 536-541.

JOO, K. M., CHUNG, Y. H., KIM, M. K., NAM, R. H., LEE, B. L., LEE, K. H. & CHA, C. I. 2004. Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors (VPAC1, VPAC2, and PAC1 receptor) in the rat brain. *Journal of Comparative Neurology,* 476**,** 388-413.

JOOSTEN, L. A., VAN DE VEERDONK, F. L., VONK, A. G., BOERMAN, O. C., KEUTER, M., FANTUZZI, G., VERSCHUEREN, I., VAN DER POLL, T., DINARELLO, C. A. & KULLBERG, B. J. 2010. Differential susceptibility to lethal endotoxaemia in mice deficient in IL-1α, IL-1β or IL-1 receptor type I. *Apmis,* 118**,** 1000-1007.

JORDENS, I., MARSMAN, M., KUIJL, C. & NEEFJES, J. 2005. Rab proteins, connecting transport and vesicle fusion. *Traffic,* 6**,** 1070-1077.

JØRGENSEN, P. F., WANG, J. E., ALMLÖF, M., THIEMERMANN, C., FOSTER, S. J., SOLBERG, R. & AASEN, A. O. 2001. Peptidoglycan and lipoteichoic acid modify monocyte phenotype in human whole blood. *Clinical and Diagnostic Laboratory Immunology,* 8**,** 515-521.

JUARRANZ, M., SANTIAGO, B., TORROBA, M., GUTIERREZ-CANAS, I., PALAO, G., GALINDO, M., ABAD, C., MARTINEZ, C., LECETA, J. & PABLOS, J. 2004. Vasoactive intestinal peptide modulates proinflammatory mediator synthesis in osteoarthritic and rheumatoid synovial cells. *Rheumatology,* 43**,** 416-422.

JUARRANZ, Y., ABAD, C., MARTINEZ, C., ARRANZ, A., GUTIERREZ-CAÑAS, I., ROSIGNOLI, F., GOMARIZ, R. P. & LECETA, J. 2005. Protective effect of vasoactive intestinal peptide on bone destruction in the collagen-induced arthritis model of rheumatoid arthritis. *Arthritis Research & Therapy,* 7**,** R1034.

JUARRANZ, Y., GUTIÉRREZ-CAÑAS, I., SANTIAGO, B., CARRIÓN, M., PABLOS, J. L. & GOMARIZ, R. P. 2008. Differential expression of vasoactive intestinal peptide and its functional receptors in

human osteoarthritic and rheumatoid synovial fibroblasts. *Arthritis & Rheumatism,* 58**,** 1086-1095.

KABANOV, D. & PROKHORENKO, I. 2010. Structural analysis of lipopolysaccharides from Gram-negative bacteria. *Biochemistry (Moscow),* 75**,** 383-404.

KAISER, P., ROTHWELL, L., GALYOV, E. E., BARROW, P. A., BURNSIDE, J. & WIGLEY, P. 2000. Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. *Microbiology,* 146**,** 3217-3226.

KALTREIDER, H. B., ICHIKAWA, S., BYRD, P. K., INGRAM, D. A., KISHIYAMA, J. L., SREEDHARAN, S. P., WARNOCK, M. L., BECK, J. M. & GOETZL, E. J. 1997. Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. *American Journal of Respiratory Cell and Molecular Biology,* 16**,** 133-144.

KAPILOFF, M. S., MATHIS, J. M., NELSON, C. A., LIN, C. R. & ROSENFELD, M. G. 1991. Calcium/calmodulin-dependent protein kinase mediates a pathway for transcriptional regulation. *Proceedings of the National Academy of Sciences,* 88**,** 3710-3714.

KARLSSON, H., LARSSON, P., WOLD, A. E. & RUDIN, A. 2004. Pattern of Cytokine Responses to Gram-Positive and Gram-Negative Commensal Bacteria is Profoundly Changed when Monocytes Differentiate into Dendritic Cells. *Scandinavian Journal of Immunology,* 59**,** 628-628.

KATO, A. & GROISMAN, E. A. 2008. The PhoQ/PhoP regulatory network of Salmonella enterica. *Bacterial Signal Transduction: Networks and Drug Targets.* Springer.

KAWAI, T. & AKIRA, S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology,* 11**,** 373-384.

KEESTRA, A. M., GODINEZ, I., XAVIER, M. N., WINTER, M. G., WINTER, S. E., TSOLIS, R. M. & BÄUMLER, A. J. 2011. Early MyD88-dependent induction of interleukin-17A expression during Salmonella colitis. *Infection and Immunity,* 79**,** 3131-3140.

KELLOGG, D. L., ZHAO, J. L., WU, Y. & JOHNSON, J. M. 2010. VIP/PACAP receptor mediation of cutaneous active vasodilation during heat stress in humans. *Journal of Applied Physiology,* 109**,** 95-100.

KIENER, P. A., DAVIS, P. M., STARLING, G. C., MEHLIN, C., KLEBANOFF, S. J., LEDBETTER, J. A. & LILES, W. C. 1997. Differential induction of apoptosis by Fas–Fas ligand interactions

in human monocytes and macrophages. *The Journal of Experimental Medicine,* 185**,** 1511-1516.

KIM, J.-E., KIM, S. Y., LIM, S. Y., KIEFF, E. & SONG, Y.-J. 2014. Role of Ca2+/Calmodulin-Dependent Kinase II–IRAK1 Interaction in LMP1-Induced NF-κB Activation. *Molecular and Cellular Biology,* 34**,** 325-334.

KIM, K. I., YAN, M., MALAKHOVA, O., LUO, J.-K., SHEN, M.-F., ZOU, W., DE LA TORRE, J. C. & ZHANG, D.-E. 2006. Ube1L and protein ISGylation are not essential for alpha/beta interferon signaling. *Molecular and Cellular Biology,* 26**,** 472-479.

KIM, W.-K., KAN, Y., GANEA, D., HART, R. P., GOZES, I. & JONAKAIT, G. M. 2000. Vasoactive intestinal peptide and pituitary adenylyl cyclase-activating polypeptide inhibit tumor necrosis factor-α production in injured spinal cord and in activated microglia via a cAMP-dependent pathway. *The Journal of Neuroscience,* 20**,** 3622-3630.

KIMBRELL, M. R., WARSHAKOON, H., CROMER, J. R., MALLADI, S., HOOD, J. D., BALAKRISHNA, R., SCHOLDBERG, T. A. & DAVID, S. A. 2008. Comparison of the immunostimulatory and proinflammatory activities of candidate Gram-positive endotoxins, lipoteichoic acid, peptidoglycan, and lipopeptides, in murine and human cells. *Immunology Letters,* 118**,** 132-141.

KIRBY, A. C., SUNDQUIST, M. & WICK, M. J. 2004. In vivo compartmentalization of functionally distinct, rapidly responsive antigen-specific T-cell populations in DNA-immunized or Salmonella enterica serovar Typhimurium-infected mice. *Infection and Immunity,* 72**,** 6390-6400.

KIRKEBØEN, K. & STRAND, O. 1999. [Nitric oxide--an important mediator in sepsis?]. *Tidsskrift for den Norske laegeforening: Tidsskrift for Praktisk Medicin, ny raekke,* 119**,** 4061-4065.

KIRSCHNING, C., UNBEHAUN, A., LAMPING, N., PFEIL, D., HERRMANN, F. & SCHUMANN, R. 1997. Control of transcriptional activation of the lipopolysaccharide binding protein (LBP) gene by proinflammatory cytokines. *Cytokines, Cellular & Molecular Therapy,* 3**,** 59-62.

KOBAYASHI, M., SAITOH, S.-I., TANIMURA, N., TAKAHASHI, K., KAWASAKI, K., NISHIJIMA, M., FUJIMOTO, Y., FUKASE, K., AKASHI-TAKAMURA, S. & MIYAKE, K. 2006. Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering. *The Journal of Immunology,* 176**,** 6211-6218.

KOJIMA, M., ITO, T., OONO, T., HISANO, T., IGARASHI, H., ARITA, Y., KAWABE, K., COY, D. H., JENSEN, R. T. & NAWATA, H. 2005. VIP attenuation of the severity of experimental pancreatitis is due to VPAC1 receptor-mediated inhibition of cytokine production. *Pancreas,* 30**,** 62-70.

KUBO, S., YAMAOKA, K., KONDO, M., YAMAGATA, K., ZHAO, J., IWATA, S. & TANAKA, Y. 2013. The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells. *Annals of the Rheumatic Diseases***,** Annrheumdis-2013-203756.

KULKA, M., SHEEN, C. H., TANCOWNY, B. P., GRAMMER, L. C. & SCHLEIMER, R. P. 2008. Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology,* 123**,** 398-410.

KUMAR, H., KAWAI, T. & AKIRA, S. 2009. Pathogen recognition in the innate immune response. *Biochem. J,* 420**,** 1-16.

KUMAR, S. & RIZVI, M. 2009. Prognostic serum tumor necrosis factor-α in paediatric patients with sepsis. *The Journal of Infection in Developing Countries,* 3**,** 437-441.

KUPPNER, M. C., SCHARNER, A., MILANI, V., VON HESLER, C., TSCHÖP, K. E., HEINZ, O. & ISSELS, R. D. 2003. Ifosfamide impairs the allostimulatory capacity of human dendritic cells by intracellular glutathione depletion. *Blood,* 102**,** 3668-3674.

KUPZ, A., BEDOUI, S. & STRUGNELL, R. A. 2014. Cellular Requirements for Systemic Control of Salmonella enterica Serovar Typhimurium Infections in Mice. *Infection and Immunity,* 82**,** 4997-5004.

KURT, A., AYGUN, A. D., GODEKMERDAN, A., KURT, A., DOGAN, Y. & YILMAZ, E. 2008. Serum IL-1 β, IL-6, IL-8, and TNF-α Levels in Early Diagnosis and Management of Neonatal Sepsis. *Mediators of Inflammation,* 2007.

KUSHNER, I. & RZEWNICKI, D. L. 1994. The acute phase response: general aspects. *Bailliere's clinical rheumatology,* 8**,** 513-530.

LABURTHE, M., COUVINEAU, A. & TAN, V. 2007. Class II G protein-coupled receptors for VIP and PACAP: structure, models of activation and pharmacology. *Peptides,* 28**,** 1631-1639.

LANGER, I. 2012. Mechanisms involved in VPAC receptors activation and regulation: lessons from pharmacological and mutagenesis studies. *Frontiers in Endocrinology,* 3.

LANGER, I., GASPARD, N. & ROBBERECHT, P. 2006. Pharmacological properties of Chinese hamster ovary cells coexpressing two vasoactive intestinal peptide receptors (hVPAC1 and hVPAC2). *British Journal of Pharmacology,* 148**,** 1051-1059.

LANGER, I., LANGLET, C. & ROBBERECHT, P. 2005. Effect of inactivating mutations on phosphorylation and internalization of the human VPAC2 receptor. *Journal of Molecular Endocrinology,* 34**,** 405-414.

LANGER, I. & ROBBERECHT, P. 2005. Mutations in the carboxy-terminus of the third intracellular loop of the human

recombinant VPAC 1 receptor impair VIP-stimulated [Ca 2+] i increase but not adenylate cyclase stimulation. *Cellular Signalling,* 17**,** 17-24.

LARA-MARQUEZ, M. L., O'DORISIO, M. S., O'DORISIO, T. M., SHAH, M. H. & KARACAY, B. 2001. Selective gene expression and activation-dependent regulation of vasoactive intestinal peptide receptor type 1 and type 2 in human T cells. *The Journal of Immunology,* 166**,** 2522-2530.

LAROCCA, L., CALAFAT, M., ROCA, V., FRANCHI, A. M. & LEIRÓS, C. P. 2007. VIP limits LPS-induced nitric oxide production through IL-10 in NOD mice macrophages. *International Immunopharmacology,* 7**,** 1343-1349.

LATIFI, S. Q., O'RIORDAN, M. A. & LEVINE, A. D. 2002. Interleukin-10 controls the onset of irreversible septic shock. *Infection and Immunity,* 70**,** 4441-4446.

LE BON, A., THOMPSON, C., KAMPHUIS, E., DURAND, V., ROSSMANN, C., KALINKE, U. & TOUGH, D. F. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *The Journal of Immunology,* 176**,** 2074-2078.

LE PECHON-VALLEE, C., MAGALON, K., RASOLONJANAHARY, R., ENJALBERT, A. & GERARD, C. 2000. Vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptides stimulate mitogen-activated protein kinase in the pituitary cell line GH4C1 by a 3', 5'. *Neuroendocrinology,* 72**,** 46.

LECETA, J., GOMARIZ, R. P., MARTINEZ, C., ABAD, C., GANEA, D. & DELGADO, M. 2000. Receptors and Transcriptional Factors Involved in the Anti-Inflammatory Activity of VIP and PACAP. *Annals of the New York Academy of Sciences,* 921**,** 92-102.

LEE, J., KRAVCHENKO, V., KIRKLAND, T., HAN, J., MACKMAN, N., MORIARTY, A., LETURCQ, D., TOBIAS, P. & ULEVITCH, R. 1993. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proceedings of the National Academy of Sciences,* 90**,** 9930-9934.

LEE, S. J., CHOI, E. K., SEO, K. W., BAE, J. U., KIM, Y. H., PARK, S. Y., OH, S. O. & KIM, C. D. 2013. 5-Lipoxygenase plays a pivotal role in endothelial adhesion of monocytes via an increased expression of Mac-1. *Cardiovascular Research,* 99**,** 724-733.

LEENDERS, A. M., DA SILVA, F. H. L., GHIJSEN, W. E. & VERHAGE, M. 2001. Rab3a is involved in transport of synaptic vesicles to the active zone in mouse brain nerve terminals. *Molecular Biology of the Cell,* 12**,** 3095-3102.

LEKKOU, A., MOUZAKI, A., SIAGRIS, D., RAVANI, I. & GOGOS, C. A. 2014. Serum lipid profile, cytokine production, and clinical

outcome in patients with severe sepsis. *Journal of Critical Care,* 29**,** 723-727.

LELIEVRE, V., FAVRAIS, G., ABAD, C., ADLE-BIASSETTE, H., LU, Y., GERMANO, P., CHEUNG-LAU, G., PISEGNA, J., GRESSENS, P. & LAWSON, G. 2007. Gastrointestinal dysfunction in mice with a targeted mutation in the gene encoding vasoactive intestinal polypeptide: a model for the study of intestinal ileus and Hirschsprung's disease. *Peptides,* 28**,** 1688-1699.

LEMAITRE, B., NICOLAS, E., MICHAUT, L., REICHHART, J.-M. & HOFFMANN, J. A. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* 86**,** 973-983.

LEVINSON, D. F., DUAN, J., OH, S., WANG, K., SANDERS, A. R., SHI, J., ZHANG, N., MOWRY, B. J., OLINCY, A. & AMIN, F. 2011. Copy number variants in schizophrenia: confirmation of five previous findings and new evidence for 3q29 microdeletions and VIPR2 duplications. *American Journal of Psychiatry*.

LEVY, M. M., FINK, M. P., MARSHALL, J. C., ABRAHAM, E., ANGUS, D., COOK, D., COHEN, J., OPAL, S. M., VINCENT, J.-L. & RAMSAY, G. 2003. 2001 sccm/esicm/accp/ats/sis international sepsis definitions conference. *Intensive Care Medicine,* 29**,** 530-538.

LI, J., SONG, C.-X., LI, Y.-P., LI, L., WEI, X.-H., WANG, J.-L. & LIU, X.-S. 2015. Rab3 is involved in cellular immune responses of the cotton bollworm, Helicoverpa armigera. *Developmental & Comparative Immunology,* 50**,** 78-86.

LINDÉN, A., CARDELL, L.-O., YOSHIHARA, S., STJÄRNE, P. & NADEL, J. A. 1998. PACAP 1-38 as an inhaled bronchodilator in guinea pigs in vivo. *Peptides,* 19**,** 93-98.

LINDEN, A., HANSSON, L., ANDERSSON, A., PALMQVIST, M., ARVIDSSON, P., LÖFDAHL, C.-G., LARSSON, P. & LÖTVALL, J. 2003. Bronchodilation by an inhaled VPAC2 receptor agonist in patients with stable asthma. *Thorax,* 58**,** 217-221.

LINDGREN, S. W., STOJILJKOVIC, I. & HEFFRON, F. 1996. Macrophage killing is an essential virulence mechanism of Salmonella typhimurium. *Proceedings of the National Academy of Sciences,* 93**,** 4197-4201.

LIPOVŠEK, S., JANŽEKOVIČ, F., LEITINGER, G. & RUPNIK, M. S. 2013. Rab3a ablation related changes in morphology of secretory vesicles in major endocrine pancreatic cells, pituitary melanotroph cells and adrenal gland chromaffin cells in mice. *General and comparative endocrinology,* 185**,** 67-79.

LIU, P., FOSTER, G., LABADIE, R., SOMOZA, E. & SHARMA, A. 2007. Pharmacokinetic interaction between voriconazole and methadone at steady state in patients on methadone therapy. *Antimicrob Agents Chemother,* 51**,** 110-8.

LIU, X., LU, R., XIA, Y. & SUN, J. 2010. Global analysis of the eukaryotic pathways and networks regulated by Salmonella typhimurium in mouse intestinal infection in vivo. *BMC genomics,* 11**,** 722.

LLEDO, P.-M., JOHANNES, L., VERNIER, P., ZOREC, R., DARCHEN, F., VINCENT, J., HENRY, J. & MASON, W. T. 1994. Rab3 proteins: key players in the control of exocytosis. *Trends in Neurosciences,* 17**,** 426-432.

LÓPEZ, F. E., DE LAS MERCEDES PESCARETTI, M., MORERO, R. & DELGADO, M. A. 2012. Salmonella Typhimurium general virulence factors: A battle of David against Goliath? *Food Research International,* 45**,** 842-851.

LU, C., VICKERS, M. F. & KERBEL, R. S. 1992. Interleukin 6: a fibroblast-derived growth inhibitor of human melanoma cells from early but not advanced stages of tumor progression. *Proceedings of the National Academy of Sciences,* 89**,** 9215-9219.

LU, Y.-C., YEH, W.-C. & OHASHI, P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine,* 42**,** 145-151.

LUTZ, E., SHEWARD, W., WEST, K., MORROW, J., FINK, G. & HARMAR, A. 1993. The VIP 2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Letters,* 334**,** 3-8.

LUU, R. A., GURNANI, K., DUDANI, R., KAMMARA, R., VAN FAASSEN, H., SIRARD, J.-C., KRISHNAN, L. & SAD, S. 2006. Delayed expansion and contraction of CD8+ T cell response during infection with virulent Salmonella typhimurium. *The Journal of Immunology,* 177**,** 1516-1525.

LUZIO, J. P., PRYOR, P. R. & BRIGHT, N. A. 2007. Lysosomes: fusion and function. *Nature Reviews Molecular Cell Biology,* 8**,** 622-632.

LUZIO, J. P., ROUS, B. A., BRIGHT, N. A., PRYOR, P. R., MULLOCK, B. M. & PIPER, R. C. 2000. Lysosome-endosome fusion and lysosome biogenesis. *Journal of Cell Science,* 113**,** 1515-1524.

LV, B., HUANG, J., YUAN, H., YAN, W., HU, G. & WANG, J. 2014. Tumor necrosis factor-α as a diagnostic marker for neonatal sepsis: a meta-analysis. *The Scientific World Journal,* 2014.

LYAKH, L. A., KOSKI, G. K., TELFORD, W., GRESS, R. E., COHEN, P. A. & RICE, N. R. 2000. Bacterial lipopolysaccharide, TNF-α, and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14+ monocytes through distinct pathways that activate NF-κB. *The Journal of Immunology,* 165**,** 3647-3655.

MA, J., ZHANG, Y.-G., XIA, Y. & SUN, J. 2010. The inflammatory cytokine tumor necrosis factor modulates the expression of

Salmonella typhimurium effector proteins. *Journal of Inflammation(London, England),* 7**,** 42-42.

MACDONALD, J., GALLEY, H. & WEBSTER, N. 2003. Oxidative stress and gene expression in sepsis. *British Journal of Anaesthesia,* 90**,** 221-232.

MACLENNAN, C. A., GONDWE, E. N., MSEFULA, C. L., KINGSLEY, R. A., THOMSON, N. R., WHITE, S. A., GOODALL, M., PICKARD, D. J., GRAHAM, S. M. & DOUGAN, G. 2008. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children. *The Journal of Clinical Investigation,* 118**,** 1553-1562.

MADAN, R., RASTOGI, R., PARASHURAMAN, S. & MUKHOPADHYAY, A. 2012. Salmonella acquires lysosome-associated membrane protein 1 (LAMP1) on phagosomes from Golgi via SipC protein-mediated recruitment of host Syntaxin6. *Journal of Biological Chemistry,* 287**,** 5574-5587.

MAHON, M. J. & SHIMADA, M. 2005. Calmodulin interacts with the cytoplasmic tails of the parathyroid hormone 1 receptor and a sub-set of class b G-protein coupled receptors. *FEBS Letters,* 579**,** 803-807.

MAJOWICZ, S. E., MUSTO, J., SCALLAN, E., ANGULO, F. J., KIRK, M., O'BRIEN, S. J., JONES, T. F., FAZIL, A. & HOEKSTRA, R. M. 2010. The global burden of nontyphoidal Salmonella gastroenteritis. *Clinical Infectious Diseases,* 50**,** 882-889.

MALAKHOVA, O. A., KIM, K. I., LUO, J. K., ZOU, W., KUMAR, K. S., FUCHS, S. Y., SHUAI, K. & ZHANG, D. E. 2006. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. *The EMBO Journal,* 25**,** 2358-2367.

MANCA, C., TSENOVA, L., BERGTOLD, A., FREEMAN, S., TOVEY, M., MUSSER, J. M., BARRY, C. E., FREEDMAN, V. H. & KAPLAN, G. 2001. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. *Proceedings of the National Academy of Sciences,* 98**,** 5752-5757.

MANČEK, M., PRISTOVŠEK, P. & JERALA, R. 2002. Identification of LPS-binding peptide fragment of MD-2, a toll-receptor accessory protein. *Biochemical and Biophysical Research Communications,* 292**,** 880-885.

MANCHESTER, K. 1996. Use of UV methods for measurement of protein and nucleic acid concentrations. *Biotechniques,* 20**,** 968.

MARTELLI, A. M., BALDINI, G., TABELLINI, G., KOTICHA, D., BAREGGI, R. & BALDINI, G. 2000. Rab3A and Rab3D control the total granule number and the fraction of granules docked at the plasma membrane in PC12 cells. *Traffic,* 1**,** 976-986.

MARTIN, G. S. 2012. Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes. *Expert Rev Anti Infect Ther*. 6, 701-706.

MARTIN, U., SCHOLLER, J., GURGEL, J., RENSHAW, B., SIMS, J. E. & GABEL, C. A. 2009. Externalization of the leaderless cytokine IL-1F6 occurs in response to lipopolysaccharide/ATP activation of transduced bone marrow macrophages. *The Journal of Immunology,* 183**,** 4021-4030.

MARTINEZ, C., ABAD, C., DELGADO, M., ARRANZ, A., JUARRANZ, M. G., RODRIGUEZ-HENCHE, N., BRABET, P., LECETA, J. & GOMARIZ, R. P. 2002. Anti-inflammatory role in septic shock of pituitary adenylate cyclase-activating polypeptide receptor. *Proceedings of the National Academy of Sciences,* 99**,** 1053-1058.

MARTINEZ, C., DELGADO, M., POZO, D., LECETA, J., CALVO, J. R., GANEA, D. & GOMARIZ, R. P. 1998. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide modulate endotoxin-induced IL-6 production by murine peritoneal macrophages. *Journal of Leukocyte Biology,* 63**,** 591-601.

MARTÍNEZ, C., JUARRANZ, Y., ABAD, C., ARRANZ, A., MIGUEL, B. G., ROSIGNOLI, F., LECETA, J. & GOMARIZ, R. P. 2005. Analysis of the role of the PAC1 receptor in neutrophil recruitment, acute-phase response, and nitric oxide production in septic shock. *Journal of Leukocyte Biology,* 77**,** 729-738.

MASTROENI, P., CLARE, S., KHAN, S., HARRISON, J., HORMAECHE, C., OKAMURA, H., KURIMOTO, M. & DOUGAN, G. 1999. Interleukin 18 Contributes to Host Resistance and Gamma Interferon Production in Mice Infected with VirulentSalmonella typhimurium. *Infection and Immunity,* 67**,** 478-483.

MASTROENI, P., GRANT, A., RESTIF, O. & MASKELL, D. 2009. A dynamic view of the spread and intracellular distribution of Salmonella enterica. *Nature Reviews Microbiology,* 7**,** 73-80.

MASTROENI, P., HARRISON, J., CHABALGOITY, J. & HORMAECHE, C. 1996. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infection and Immunity,* 64**,** 189-196.

MASTROENI, P., VAZQUEZ-TORRES, A., FANG, F. C., XU, Y., KHAN, S., HORMAECHE, C. E. & DOUGAN, G. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *The Journal of Experimental Medicine,* 192**,** 237-248.

MATHIOUDAKIS, A., CHATZIMAVRIDOU-GRIGORIADOU, V., EVANGELOPOULOU, E. & MATHIOUDAKIS, G. 2013. Vasoactive

intestinal peptide inhaled agonists: potential role in respiratory therapeutics. *Hippokratia,* 17**,** 12.

MATOT, I. & SPRUNG, C. L. 2001. Definition of sepsis. *Intensive care medicine,* 27**,** S3-S9.

MATTNER, F., OZMEN, L., PODLASKI, F. J., WILKINSON, V. L., PRESKY, D. H., GATELY, M. K. & ALBER, G. 1997. Treatment with homodimeric interleukin-12 (IL-12) p40 protects mice from IL-12-dependent shock but not from tumor necrosis factor alpha-dependent shock. *Infection and Immunity,* 65**,** 4734-4737.

MATTSON, M. P., LAFERLA, F. M., CHAN, S. L., LEISSRING, M. A., SHEPEL, P. N. & GEIGER, J. D. 2000. Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. *Trends in Neurosciences,* 23**,** 222-229.

MATTSSON, E., VERHAGE, L., ROLLOF, J., FLEER, A., VERHOEF, J. & VAN DIJK, H. 1993. Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumour necrosis factor-α, interleukin-1β and interleukin-6. *FEMS Immunology & Medical Microbiology,* 7**,** 281-287.

MAXFIELD, F. R. & MCGRAW, T. E. 2004. Endocytic recycling. *Nature reviews Molecular Cell Biology,* 5**,** 121-132.

MCCORMICK, B. A., PARKOS, C. A., COLGAN, S. P., CARNES, D. K. & MADARA, J. L. 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. *The Journal of Immunology,* 160**,** 455-466.

MCCULLOCH, D., MACKENZIE, C., JOHNSON, M., ROBERTSON, D., HOLLAND, P., RONALDSON, E., LUTZ, E. & MITCHELL, R. 2002. Additional signals from VPAC/PAC family receptors. *Biochemical Society Transactions,* 30**,** 441-446.

MCDERMOTT, P. F., CIACCI-WOOLWINE, F., SNIPES, J. A. & MIZEL, S. B. 2000. High-affinity interaction between gram-negative flagellin and a cell surface polypeptide results in human monocyte activation. *Infection and Immunity,* 68**,** 5525-5529.

MCDONALD, T. P., DINNIS, D. M., MORRISON, C. F. & HARMAR, A. J. 1998. Desensitization of the Human Vasoactive Intestinal Peptide Receptor (hVIP2/PACAP R): Evidence for Agonist-Induced Receptor Phosphorylation and Internalizationa. *Annals of the New York Academy of Sciences,* 865**,** 64-72.

MCGEACHY, M. J. & MCSORLEY, S. J. 2012. Microbial-induced Th17: superhero or supervillain? *The Journal of Immunology,* 189**,** 3285-3291.

MCSORLEY, S. J., ASCH, S., COSTALONGA, M., REINHARDT, R. L. & JENKINS, M. K. 2002. Tracking Salmonella-specific CD4 T cells in vivo reveals a local mucosal response to a disseminated infection. *Immunity,* 16**,** 365-377.

MELAMED, A. & SORVILLO, F. J. 2009. The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. *Critical Care,* 13**,** R28.

MENG, A., ZHANG, X. & SHI, Y. 2014. Role of p38 MAPK and STAT3 in lipopolysaccharide-stimulated mouse alveolar macrophages. *Experimental and Therapeutic Medicine,* 8**,** 1772-1776.

MIAO, E. A., LEAF, I. A., TREUTING, P. M., MAO, D. P., DORS, M., SARKAR, A., WARREN, S. E., WEWERS, M. D. & ADEREM, A. 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nature Immunology,* 11**,** 1136-1142.

MICHEL, T., REICHHART, J.-M., HOFFMANN, J. A. & ROYET, J. 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature,* 414**,** 756-759.

MILLEDGE, J., CALIS, J., GRAHAM, S., PHIRI, A., WILSON, L., SOKO, D., MBVWINJI, M., WALSH, A. L., ROGERSON, S. & MOLYNEUX, E. 2005. Aetiology of neonatal sepsis in Blantyre, Malawi: 1996–2001. *Annals of Tropical Paediatrics,* 25**,** 101-110.

MILLER, S. & MEKALANOS, J. 1990. Constitutive expression of the phoP regulon attenuates Salmonella virulence and survival within macrophages. *Journal of Bacteriology,* 172**,** 2485-2490.

MIROLD, S., RABSCH, W., TSCHÄPE, H. & HARDT, W.-D. 2001. Transfer of the Salmonella type III effector sopE between unrelated phage families. *Journal of Molecular Biology,* 312**,** 7-16.

MITCHELL, E. K., MASTROENI, P., KELLY, A. P. & TROWSDALE, J. 2004. Inhibition of cell surface MHC class II expression by Salmonella. *European Journal of Immunology,* 34**,** 2559-2567.

MITTRÜCKER, H.-W. & KAUFMANN, S. 2000. Immune response to infection with Salmonella typhimurium in mice. *Journal of Leukocyte Biology,* 67**,** 457-463.

MIYAKE, K. 2004. Innate recognition of lipopolysaccharide by Toll-like receptor 4–MD-2. *Trends in Microbiology,* 12**,** 186-192.

MOGENSEN, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews,* 22**,** 240-273.

MOLOFSKY, A. B., BYRNE, B. G., WHITFIELD, N. N., MADIGAN, C. A., FUSE, E. T., TATEDA, K. & SWANSON, M. S. 2006. Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. *The Journal of Experimental Medicine,* 203**,** 1093-1104.

MONACK, D. M., NAVARRE, W. W. & FALKOW, S. 2001. Salmonella-induced macrophage death: the role of caspase-1 in death and inflammation. *Microbes and Infection,* 3**,** 1201-1212.

MOORE, K. W., DE WAAL MALEFYT, R., COFFMAN, R. L. & O'GARRA, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology,* 19**,** 683-765.

MORELL, M., SOUZA-MOREIRA, L. & GONZALEZ-REY, E. 2012. VIP in neurological diseases: more than a neuropeptide. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* 12**,** 323-332.

MORGENTHALER, N. G. & KOSTRZEWA, M. 2015. Rapid identification of pathogens in positive blood culture of patients with sepsis: review and meta-analysis of the performance of the sepsityper kit. *International Journal of Microbiology,* 2015.

MORICE, A., UNWIN, R. & SEVER, P. 1984. Vasoactive intestinal peptide as a bronchodilator in asthmatic subjects. *Peptides,* 5**,** 439-440.

MURASE, T., YAMADA, M., MUTO, T., MATSUSHIMA, A. & YAMAI, S. 2000. Fecal Excretion of Salmonella entericaSerovar Typhimurium Following a Food-Borne Outbreak. *Journal of Clinical Microbiology,* 38**,** 3495-3497.

MURPHY, K. M., OUYANG, W., FARRAR, J. D., YANG, J., RANGANATH, S., ASNAGLI, H., AFKARIAN, M. & MURPHY, T. L. 2000. Signaling and transcription in T helper development. *Annual Review of Immunology,* 18**,** 451-494.

MURRAY, R. Z., KAY, J. G., SANGERMANI, D. G. & STOW, J. L. 2005. A role for the phagosome in cytokine secretion. *Science,* 310**,** 1492-1495.

MURTHY, K. S., MAHAVADI, S., HUANG, J., ZHOU, H. & SRIWAI, W. 2008. Phosphorylation of GRK2 by PKA augments GRK2-mediated phosphorylation, internalization, and desensitization of VPAC2 receptors in smooth muscle. *American Journal of Physiology-Cell Physiology,* 294**,** C477-C487.

MUSGROVE, M., JONES, D., NORTHCUTT, J., COX, N., HARRISON, M., FEDORKA-CRAY, P. & LADELY, S. 2006. Antimicrobial resistance in Salmonella and Escherichia coli isolated from commercial shell eggs. *Poultry Science,* 85**,** 1665-1669.

MUTAMBA, S., ALLISON, A., MAHIDA, Y., BARROW, P. & FOSTER, N. 2012. Expression of IL-1Rrp2 by human myelomonocytic cells is unique to DCs and facilitates DC maturation by IL-1F8 and IL-1F9. *European Journal of Immunology,* 42**,** 607-617.

NAGAI, Y., AKASHI, S., NAGAFUKU, M., OGATA, M., IWAKURA, Y., AKIRA, S., KITAMURA, T., KOSUGI, A., KIMOTO, M. & MIYAKE,

K. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature Immunology,* 3**,** 667-672.

NAKAE, H., ENDO, S., INADA, K., TAKAKUWA, T. & KASAI, T. 1996. Changes in adhesion molecule levels in sepsis. *Research Communications in Molecular Pathology and Pharmacology,* 91**,** 329-338.

NAKAHIRA, M., TOMURA, M., IWASAKI, M., AHN, H.-J., BIAN, Y., HAMAOKA, T., OHTA, T., KURIMOTO, M. & FUJIWARA, H. 2001. An absolute requirement for STAT4 and a role for IFN-γ as an amplifying factor in IL-12 induction of the functional IL-18 receptor complex. *The Journal of Immunology,* 167**,** 1306-1312.

NAKAIRA-TAKAHAGI, E., GOLIM, M. A., BANNWART, C. F., PUCCIA, R. & PERAÇOLI, M. T. 2011. Interactions between TLR2, TLR4, and mannose receptors with gp43 from Paracoccidioides brasiliensis induce cytokine production by human monocytes. *Medical Mycology,* 49**,** 694-703.

NAKAMURA, K., OKAMURA, H., WADA, M., NAGATA, K. & TAMURA, T. 1989. Endotoxin-induced serum factor that stimulates gamma interferon production. *Infection and Immunity,* 57**,** 590-595.

NATHAN, A. & SINGER, M. 1999. The oxygen trail: tissue oxygenation. *British Medical Bulletin,* 55**,** 96-108.

NGKELO, A., MEJA, K., YEADON, M., ADCOCK, I. & KIRKHAM, P. A. 2012. LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and G i α dependent PI-3kinase signalling. *Journal of Inflammation,* 9**,** 1.

NGUYEN, H. B., RIVERS, E. P., ABRAHAMIAN, F. M., MORAN, G. J., ABRAHAM, E., TRZECIAK, S., HUANG, D. T., OSBORN, T., STEVENS, D. & TALAN, D. A. 2006. Severe sepsis and septic shock: review of the literature and emergency department management guidelines. *Annals of Emergency Medicine,* 48**,** 54. e1.

OGUNLEYE, V., OGUNLEYE, A., AJUWAPE, A., OLAWOLE, O. & ADETOSOYE, A. 2006. Childhood septicaemia due to salmonella species in Ibadan, Nigeria. *African Journal of Biomedical Research,* 8**,** 131-134.

OH, H. 1998. Emerging therapies for sepsis and septic shock. *Annals-Academy of Medicine Singapore,* 27**,** 738-743.

OHLSSON, K., BJÖRK, P., BERGENFELDT, M., HAGEMAN, R. & THOMPSON, R. C. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature*. 6301, 550-502.

OHMORI, Y. & HAMILTON, T. A. 1992. Ca2+ and calmodulin selectively regulate lipopolysaccharide-inducible cytokine mRNA expression in murine peritoneal macrophages. *The Journal of Immunology,* 148**,** 538-545.

OKAHASHI, N., YAMAMOTO, M., VANCOTT, J. L., CHATFIELD, S. N., ROBERTS, M., BLUETHMANN, H., HIROI, T., KIYONO, H. & MCGHEE, J. R. 1996. Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant Salmonella strain or cholera toxin reveals that CD4+ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses. *Infection and Immunity,* 64**,** 1516-1525.

OKAMURA, H., KASHIWAMURA, S.-I., TSUTSUI, H., YOSHIMOTO, T. & NAKANISHI, K. 1998. Regulation of interferon-γ production by IL-12 and IL-18. *Current Opinion in Immunology,* 10**,** 259-264.

OKUSAWA, S., GELFAND, J. A., IKEJIMA, T., CONNOLLY, R. & DINARELLO, C. 1988. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *Journal of Clinical Investigation,* 81**,** 1162.

OPAL, S. M. 2007. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. *International Journal of Medical Microbiology,* 297**,** 365-377.

OPPMANN, B., LESLEY, R., BLOM, B., TIMANS, J. C., XU, Y., HUNTE, B., VEGA, F., YU, N., WANG, J. & SINGH, K. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity,* 13**,** 715-725.

PÅLSSON-MCDERMOTT, E. M. & O'NEILL, L. A. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology,* 113**,** 153-162.

PARAMESWARAN, N. & PATIAL, S. 2010. Tumor necrosis factor-α signaling in macrophages. *Critical Reviews™ in Eukaryotic Gene Expression,* 20.

PARK, J.-B. 1996. Dissociation of Rab3A by Ca (2+)/calmodulin from different kinds of synaptosomal membranes. *Experimental & Molecular Medicine,* 28**,** 147-152.

PARK, J.-B., LEE, J.-Y. & KIM, J.-W. 1999. Dissociation of RalA from synaptic membranes by Ca 2+/calmodulin. *Biochemical and Biophysical Research Communications,* 263**,** 765-769.

PATHAN, N., WILLIAMS, E. J., ORAGUI, E. E., STEPHENS, A. C. & LEVIN, M. 2005. Changes in the interleukin-6/soluble interleukin-6 receptor axis in meningococcal septic shock*. *Critical Care Medicine,* 33**,** 1839-1844.

PAULIN, S. M., JAGANNATHAN, A., CAMPBELL, J., WALLIS, T. S. & STEVENS, M. P. 2007. Net replication of Salmonella enterica serovars Typhimurium and Choleraesuis in porcine intestinal mucosa and nodes is associated with their differential virulence. *Infection and Immunity,* 75**,** 3950-3960.

PERALTA, G., RODRÍGUEZ-LERA, M. J., GARRIDO, J. C., ANSORENA, L. & ROIZ, M. P. 2006. Time to positivity in blood cultures of adults with S treptococcus pneumoniae bacteremia. *BMC Infectious Diseases,* 6**,** 1.

PERLMAN, H., PAGLIARI, L. J., GEORGANAS, C., MANO, T., WALSH, K. & POPE, R. M. 1999. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *The Journal of Experimental Medicine,* 190**,** 1679-1688.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research,* 29**,** e45-e45.

PIERCE, K. L., FUJINO, H., SRINIVASAN, D. & REGAN, J. W. 1999. Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *Journal of Biological Chemistry,* 274**,** 35944-35949.

PIERCE, K. L., PREMONT, R. T. & LEFKOWITZ, R. J. 2002. Seven-transmembrane receptors. *Nature Reviews Molecular Cell Biology,* 3**,** 639-650.

PIETILÄ, T. E., VECKMAN, V., KYLLÖNEN, P., LÄHTEENMÄKI, K., KORHONEN, T. K. & JULKUNEN, I. 2005. Activation, cytokine production, and intracellular survival of bacteria in Salmonella-infected human monocyte-derived macrophages and dendritic cells. *Journal of Leukocyte Biology,* 78**,** 909-920.

POPOFF, M. Y., BOCKEMUHL , J., BRENNER, F. W. & GHEESLING, L.L. (ed.) 2001. *Supplement 2000 (no. 44) to the Kauffmann-White scheme*.

POZO, D. & DELGADO, M. 2004. The many faces of VIP in neuroimmunology: a cytokine rather a neuropeptide? *The FASEB Journal,* 18**,** 1325-1334.

POZO, D., DELGADO, M., MARTINEZ, C., GOMARIZ, R. P., GUERRERO, J. M. & CALVO, J. R. 1997. Functional characterization and mRNA expression of pituitary adenylate cyclase activating polypeptide (PACAP) type I receptors in rat peritoneal macrophages. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1359**,** 250-262.

PRICE, J. D., SIMPFENDORFER, K. R., MANTENA, R. R., HOLDEN, J., HEATH, W. R., VAN ROOIJEN, N., STRUGNELL, R. A. & WIJBURG, O. L. 2007. Gamma interferon-independent effects of interleukin-12 on immunity to Salmonella enterica serovar Typhimurium. *Infection and Immunity,* 75**,** 5753-5762.

QIAN, B. F., HAMMARSTRÖM, M. L. & DANIELSSON, Å. 2001. Differential expression of vasoactive intestinal polypeptide

receptor 1 and 2 mRNA in murine intestinal T lymphocyte subtypes. *Journal of Neuroendocrinology,* 13**,** 818-825.

QIU, P., CUI, X., SUN, J., WELSH, J., NATANSON, C. & EICHACKER, P. Q. 2013. Anti-Tumor Necrosis Factor Therapy is Associated with Improved Survival in Clinical Sepsis Trials: A Meta-analysis. *Critical Care Medicine,* 41.

RAAD, I., HANNA, H. & MAKI, D. 2007. Intravascular catheter-related infections: advances in diagnosis, prevention, and management. *The Lancet Infectious Diseases,* 7**,** 645-657.

RACIOPPI, L. & MEANS, A. R. 2012. Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology. *Journal of Biological Chemistry,* 287**,** 31658-31665.

RACIOPPI, L., NOELDNER, P. K., LIN, F., ARVAI, S. & MEANS, A. R. 2012. Calcium/calmodulin-dependent protein kinase kinase 2 regulates macrophage-mediated inflammatory responses. *Journal of Biological Chemistry,* 287**,** 11579-11591.

RAETZ, C. R., GUAN, Z., INGRAM, B. O., SIX, D. A., SONG, F., WANG, X. & ZHAO, J. 2009. Discovery of new biosynthetic pathways: the lipid A story. *Journal of Lipid Research,* 50**,** S103-S108.

RAMADORI, G., MEYER ZUM BUSCHENFELDE, K.-H., TOBIAS, P., MATHISON, J. & ULEVITCH, R. 1990. Biosynthesis of lipopolysaccharide-binding protein in rabbit hepatocytes. *Pathobiology,* 58**,** 89-94.

RANDOLPH, A. G. & MCCULLOH, R. J. 2014. Pediatric sepsis: important considerations for diagnosing and managing severe infections in infants, children, and adolescents. *Virulence,* 5**,** 179-189.

RANGON, C.-M., GOURSAUD, S., MEDJA, F., LELIÈVRE, V., MOUNIEN, L., HUSSON, I., BRABET, P., JÉGOU, S., JANET, T. & GRESSENS, P. 2005. VPAC2 receptors mediate vasoactive intestinal peptide-induced neuroprotection against neonatal excitotoxic brain lesions in mice. *Journal of Pharmacology and Experimental Therapeutics,* 314**,** 745-752.

RATHINAM, V. A., VANAJA, S. K., WAGGONER, L., SOKOLOVSKA, A., BECKER, C., STUART, L. M., LEONG, J. M. & FITZGERALD, K. A. 2012. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell,* 150**,** 606-619.

RAU, F. C., DIETER, J., LUO, Z., PRIEST, S. O. & BAUMGARTH, N. 2009. B7-1/2 (CD80/CD86) direct signaling to B cells enhances IgG secretion. *The Journal of Immunology,* 183**,** 7661-7671.

REFAI, E., JONSSON, C., ANDERSSON, M., JACOBSSON, H., LARSSON, S., KOGNER, P. & HASSAN, M. 1999. Biodistribution of liposomal 131 I-VIP in rat using gamma camera. *Nuclear Medicine and Biology,* 26**,** 931-936.

REGAZZI, R. 2007. *Molecular mechanisms of exocytosis*, Springer.

REGAZZI, R., KIKUCHI, A., TAKAI, Y. & WOLLHEIM, C. 1992. The small GTP-binding proteins in the cytosol of insulin-secreting cells are complexed to GDP dissociation inhibitor proteins. *Journal of Biological Chemistry,* 267**,** 17512-17519.

REN, M., XU, G., ZENG, J., DE LEMOS-CHIARANDINI, C., ADESNIK, M. & SABATINI, D. D. 1998. Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proceedings of the National Academy of Sciences,* 95**,** 6187-6192.

REUBI, J. C. 2000. In vitro evaluation of VIP/PACAP receptors in healthy and diseased human tissues: clinical implications. *Annals of the New York Academy of Sciences,* 921**,** 1-25.

RICCIOTTI, E. & FITZGERALD, G. A. 2011. Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 31**,** 986-1000.

RICHER, E., PRENDERGAST, C., ZHANG, D.-E., QURESHI, S. T., VIDAL, S. M. & MALO, D. 2010. N-ethyl-N-nitrosourea–induced mutation in ubiquitin-specific peptidase 18 causes hyperactivation of IFN-αβ signaling and suppresses STAT4-induced IFN-γ production, resulting in increased susceptibility to Salmonella typhimurium. *The Journal of Immunology,* 185**,** 3593-3601.

RICHTER-DAHLFORS, A., BUCHAN, A. M. & FINLAY, B. B. 1997. Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *The Journal of Experimental Medicine,* 186**,** 569-580.

RINK, J., GHIGO, E., KALAIDZIDIS, Y. & ZERIAL, M. 2005. Rab conversion as a mechanism of progression from early to late endosomes. *Cell,* 122**,** 735-749.

ROMAGNANI, S. 1999. Th1/Th2 cells. *Inflammatory Bowel Diseases,* 5**,** 285-294.

ROMERO, I. G., PAI, A. A., TUNG, J. & GILAD, Y. 2014. RNA-seq: impact of RNA degradation on transcript quantification. *BMC Biology,* 12**,** 42.

ROSENGART, M. R., ARBABI, S., BAUER, G. J., GARCIA, I., JELACIC, S. & MAIER, R. V. 2002. The actin cytoskeleton: an essential component for enhanced TNFα production by adherent monocytes. *Shock,* 17**,** 109-113.

ROSENGART, M. R., ARBABI, S., GARCIA, I. & MAIER, R. V. 2000. INTERACTIONS OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASES (CaMK) AND EXTRACELLULAR-REGULATED KINASE (ERK) IN MONOCYTE ADHERENCE AND TNF [alpha] PRODUCTION. *Shock,* 13**,** 183-189.

ROSSOL, M., PIERER, M., RAULIEN, N., QUANDT, D., MEUSCH, U., ROTHE, K., SCHUBERT, K., SCHÖNEBERG, T., SCHAEFER, M. & KRÜGEL, U. 2012. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature Communications,* 3**,** 1329.

ROST, B., HANF, G., OHNEMUS, U., OTTO-KNAPP, R., GRONEBERG, D. A., KUNKEL, G. & NOGA, O. 2005. Monocytes of allergics and non-allergics produce, store and release the neurotrophins NGF, BDNF and NT-3. *Regulatory Peptides,* 124**,** 19-25.

ROTTA, G., EDWARDS, E. W., SANGALETTI, S., BENNETT, C., RONZONI, S., COLOMBO, M. P., STEINMAN, R. M., RANDOLPH, G. J. & RESCIGNO, M. 2003. Lipopolysaccharide or whole bacteria block the conversion of inflammatory monocytes into dendritic cells in vivo. *The Journal of Experimental Medicine,* 198**,** 1253-1263.

RUDOLF, R., SALM, T., RUSTOM, A. & GERDES, H.-H. 2001. Dynamics of immature secretory granules: role of cytoskeletal elements during transport, cortical restriction, and F-actin-dependent tethering. *Molecular Biology of the Cell,* 12**,** 1353-1365.

RYDSTRÖM, A. & WICK, M. J. 2007. Monocyte recruitment, activation, and function in the gut-associated lymphoid tissue during oral Salmonella infection. *The Journal of Immunology,* 178**,** 5789-5801.

RYDSTRÖM, A. & WICK, M. J. 2009. Monocyte and neutrophil recruitment during oral Salmonella infection is driven by MyD88-derived chemokines. *European Journal of Immunology,* 39**,** 3019-3030.

SABAT, R. 2010. IL-10 family of cytokines. *Cytokine & Growth Factor Reviews,* 21**,** 315-324.

SAD, S., DUDANI, R., GURNANI, K., RUSSELL, M., VAN FAASSEN, H., FINLAY, B. & KRISHNAN, L. 2008. Pathogen proliferation governs the magnitude but compromises the function of CD8 T cells. *The Journal of Immunology,* 180**,** 5853-5861.

SADE, K., SCHWARTZ, D., WOLMAN, Y., SCHWARTZ, I., CHERNICHOVSKI, T., BLUM, M., BRAZOWSKI, E., KEYNAN, S., RAZ, I. & BLANTZ, R. C. 1999. Time course of lipopolysaccharide-induced nitric oxide synthase mRNA expression in rat glomeruli. *Journal of Laboratory and Clinical Medicine,* 134**,** 471-477.

SAID, S. I. & MUTT, V. 1970. Polypeptide with broad biological activity: isolation from small intestine. *Science,* 169**,** 1217-1218.

SALCEDO, S. P. & HOLDEN, D. W. 2003. SseG, a virulence protein that targets Salmonella to the Golgi network. *The EMBO Journal,* 22**,** 5003-5014.

SALYERS, A. A. A. W., D.D. 2002. Salmonella species. In Bacterial Pathogenesis: A Molecular Approach

SANLIOGLU, A. D., KARACAY, B., BALCI, M. K., GRIFFITH, T. S. & SANLIOGLU, S. 2012. Therapeutic potential of VIP vs PACAP in diabetes. *Journal of Molecular Endocrinology,* 49**,** R157-R167.

SASAKI, D. T., DUMAS, S. E. & ENGLEMAN, E. G. 1987. Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. *Cytometry,* 8**,** 413-420.

SCHAUMANN, R., SCHLICK, T., SCHAPER, M. & SHAH, P. M. 1997. Is TNF-α a prognostic factor in patients with sepsis? *Clinical Microbiology and Infection,* 3**,** 24-31.

SCHERLE, P. A., JONES, E. A., FAVATA, M. F., DAULERIO, A. J., COVINGTON, M. B., NURNBERG, S. A., MAGOLDA, R. L. & TRZASKOS, J. M. 1998. Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. *The Journal of Immunology,* 161**,** 5681-5686.

SCHLUMBERGER, M. & HARDT, W.-D. 2005. Triggered phagocytosis by Salmonella: bacterial molecular mimicry of RhoGTPase activation/deactivation. *Bacterial Virulence Factors and Rho GTPases.* Springer.

SCHLÜTER, O. M., BASU, J., SÜDHOF, T. C. & ROSENMUND, C. 2006. Rab3 superprimes synaptic vesicles for release: implications for short-term synaptic plasticity. *The Journal of Neuroscience,* 26**,** 1239-1246.

SCHLÜTER, O. M., KHVOTCHEV, M., JAHN, R. & SÜDHOF, T. C. 2002. Localization versus function of Rab3 proteins Evidence for a common regulatory role in controlling fusion. *Journal of Biological Chemistry,* 277**,** 40919-40929.

SCHLÜTER, O. M., SCHMITZ, F., JAHN, R., ROSENMUND, C. & SÜDHOF, T. C. 2004. A complete genetic analysis of neuronal Rab3 function. *The Journal of Neuroscience,* 24**,** 6629-6637.

SCHMIDT-WEBER, C., ALEXANDER, S., HENAULT, L., JAMES, L. & LICHTMAN, A. 1999. IL-4 enhances IL-10 gene expression in murine Th2 cells in the absence of TCR engagement. *The Journal of Immunology,* 162**,** 238-244.

SCHNEIDER-BRACHERT, W., TCHIKOV, V., NEUMEYER, J., JAKOB, M., WINOTO-MORBACH, S., HELD-FEINDT, J., HEINRICH, M., MERKEL, O., EHRENSCHWENDER, M. & ADAM, D. 2004. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity,* 21**,** 415-428.

SCHROMM, A. B., LIEN, E., HENNEKE, P., CHOW, J. C., YOSHIMURA, A., HEINE, H., LATZ, E., MONKS, B. G., SCHWARTZ, D. A. & MIYAKE, K. 2001. Molecular Genetic Analysis of an Endotoxin

Nonresponder Mutant Cell Line A Point Mutation in a Conserved Region of Md-2 Abolishes Endotoxin-Induced Signaling. *The Journal of Experimental Medicine,* 194**,** 79-88.

SCHULTE, W., BERNHAGEN, J. & BUCALA, R. 2013. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets—an updated view. *Mediators of Inflammation,* 2013.

SCHULZ, S., RÖCKEN, C., MAWRIN, C., WEISE, W., HÖLLT, V. & SCHULZ, S. 2004. Immunocytochemical identification of VPAC1, VPAC2, and PAC1 receptors in normal and neoplastic human tissues with subtype-specific antibodies. *Clinical Cancer Research,* 10**,** 8235-8242.

SCHULZ, S. M., KÖHLER, G., HOLSCHER, C., IWAKURA, Y. & ALBER, G. 2008. IL-17A is produced by Th17, γδ T cells and other CD4− lymphocytes during infection with Salmonella enterica serovar Enteritidis and has a mild effect in bacterial clearance. *International Immunology,* 20**,** 1129-1138.

SECHER, T., VASSEUR, V., POISSON, D. M., MITCHELL, J. A., CUNHA, F. Q., ALVES-FILHO, J. C. & RYFFEL, B. 2009. Crucial role of TNF receptors 1 and 2 in the control of polymicrobial sepsis. *The Journal of Immunology,* 182**,** 7855-7864.

SEEPERSADSINGH, N. & ADESIYUN, A. 2003. Prevalence and antimicrobial resistance of Salmonella spp. in pet mammals, reptiles, fish aquarium water, and birds in Trinidad. *Journal of Veterinary Medicine, Series B,* 50**,** 488-493.

SEINO, S. & SHIBASAKI, T. 2005. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiological Reviews,* 85**,** 1303-1342.

SETHI, V., ÖNYÜKSEL, H. & RUBINSTEIN, I. 2005. Liposomal vasoactive intestinal peptide. *Methods in Enzymology,* 391**,** 377-395.

SHANKAR-HARI, M., SPENCER, J., SEWELL, W. A., ROWAN, K. M. & SINGER, M. 2012. Bench-to-bedside review: Immunoglobulin therapy for sepsis-biological plausibility from a critical care perspective. *Crit Care,* 16**,** 206.

SHETZLINE, M. A., WALKER, J. K., VALENZANO, K. J. & PREMONT, R. T. 2002. Vasoactive Intestinal Polypeptide Type-1 Receptor Regulation DESENSITIZATION, PHOSPHORYLATION, AND SEQUESTRATION. *Journal of Biological Chemistry,* 277**,** 25519-25526.

SHI, X.-Z. & SARNA, S. K. 2009. Homeostatic and therapeutic roles of VIP in smooth muscle function: myo-neuroimmune interactions. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 297**,** G716-G725.

SHIMAOKA, M. & PARK, E. 2008. Advances in understanding sepsis. *European Journal of Anaesthesiology,* 25**,** 146-153.

SHIMAUCHI, H., OGAWA, T., OKUDA, K., KUSUMOTO, Y. & OKADA, H. 1999. Autoregulatory effect of interleukin-10 on proinflammatory cytokine production by Porphyromonas gingivalis lipopolysaccharide-tolerant human monocytes. *Infection and Immunity,* 67**,** 2153-2159.

SHIMONI, Z., PITLIK, S., LEIBOVICI, L., SAMRA, Z., KONIGSBERGER, H., DRUCKER, M., AGMON, V., ASHKENAZI, S. & WEINBERGER, M. 1999. Nontyphoid Salmonella bacteremia: age-related differences in clinical presentation, bacteriology, and outcome. *Clinical Infectious Diseases,* 28**,** 822-827.

SHIN, D.-I., BANNING, U., KIM, Y.-M., VERHEYEN, J., HANNEN, M., BÖNIG, H. & KÖRHOLZ, D. 1999. Interleukin 10 inhibits TNF-alpha production in human monocytes independently of interleukin 12 and interleukin 1 beta. *Immunological Investigations,* 28**,** 165-175.

SHIN, S. & BRODSKY, I. E. The inflammasome: Learning from bacterial evasion strategies. Seminars in immunology, 2015. *Elsevier*.

SIDHU, R. S., CLOUGH, R. R. & BHULLAR, R. P. 2003a. Ca 2+/calmodulin binds and dissociates K-RasB from membrane. *Biochemical and Biophysical Research Communications,* 304**,** 655-660.

SIDHU, R. S., CLOUGH, R. R. & BHULLAR, R. P. 2003b. Rab3 Small GTP—Binding Proteins: Regulation by Calcium/Calmodulin. *Signal Transduction and Cardiac Hypertrophy.* Springer.

SIMS, J. E. & SMITH, D. E. 2010. The IL-1 family: regulators of immunity. *Nature Reviews Immunology,* 10**,** 89-102.

SINISTRO, A., CIAPRINI, C., NATOLI, S., SUSSARELLO, E., CARDUCCI, F. C., ALMERIGHI, C., CAPOZZI, M., BOLACCHI, F., ROCCHI, G. & BERGAMINI, A. 2007. Lipopolysaccharide desensitizes monocytes–macrophages to CD40 ligand stimulation. *Immunology,* 122**,** 362-370.

SKELIN, M. & RUPNIK, M. 2011. cAMP increases the sensitivity of exocytosis to Ca 2+ primarily through protein kinase A in mouse pancreatic beta cells. *Cell Calcium,* 49**,** 89-99.

SMALLEY, S., BARROW, P. & FOSTER, N. 2009. Immunomodulation of innate immune responses by vasoactive intestinal peptide (VIP): its therapeutic potential in inflammatory disease. *Clinical & Experimental Immunology,* 157**,** 225-234.

SONG, J., DUNCAN, M. J., LI, G., CHAN, C., GRADY, R., STAPLETON, A. & ABRAHAM, S. N. 2007. A novel TLR4-mediated signaling pathway leading to IL-6 responses in human bladder epithelial cells. *PLoS Pathog,* 3**,** e60-e60.

SONG, S.-K., KARL, I. E., ACKERMAN, J.-L. & HOTCHKISS, R. S. 1993. Increased intracellular Ca2+: a critical link in the

pathophysiology of sepsis? *Proceedings of the National Academy of Sciences,* 90**,** 3933-3937.

SÖNNICHSEN, B., DE RENZIS, S., NIELSEN, E., RIETDORF, J. & ZERIAL, M. 2000. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *The Journal of Cell Biology,* 149**,** 901-914.

SOUWER, Y., GRIEKSPOOR, A., JORRITSMA, T., DE WIT, J., JANSSEN, H., NEEFJES, J. & VAN HAM, S. M. 2009. B cell receptor-mediated internalization of salmonella: a novel pathway for autonomous B cell activation and antibody production. *The Journal of Immunology,* 182**,** 7473-7481.

SOUZA-FONSECA-GUIMARAES, F., CAVAILLON, J.-M. & ADIB-CONQUY, M. 2013. Bench-to-bedside review: Natural killer cells in sepsis-guilty or not guilty? *Crit Care,* 17**,** 235.

SPITTLER, A., RAZENBERGER, M., KUPPER, H., KAUL, M., HACKL, W., BOLTZ-NITULESCU, G., FÜGGER, R. & ROTH, E. 2000. Relationship between interleukin-6 plasma concentration in patients with sepsis, monocyte phenotype, monocyte phagocytic properties, and cytokine production. *Clinical Infectious Diseases,* 31**,** 1338-1342.

SPOONER, C. E., MARKOWITZ, N. P. & SARAVOLATZ, L. D. 1992. The role of tumor necrosis factor in sepsis. *Clinical Immunology and Immunopathology,* 62**,** S11-S17.

SPROUL, T. W., CHENG, P. C., DYKSTRA, M. L. & PIERCE, S. K. 2000. A role for MHC class II antigen processing in B cell development. *International Reviews of Immunology,* 19**,** 139-155.

SREEDHARAN, S. P., PATEL, D. R., XIA, M., ICHIKAWA, S. & GOETZL, E. J. 1994. Human Vasoactive Intestinal Peptide 1 Receptors Expressed by Stable Transfectants Couple to 2 Distinct Signaling Pathways. *Biochemical and Biophysical Research Communications,* 203**,** 141-148.

SRINIVASAN, A., FOLEY, J. & MCSORLEY, S. J. 2004. Massive number of antigen-specific CD4 T cells during vaccination with live attenuated Salmonella causes interclonal competition. *The Journal of Immunology,* 172**,** 6884-6893.

SRISANGTHONG, P., WONGSA, A., KITTIWORAWITKUL, P. & WATTANATHUM, A. 2013. Early IL-6 response in sepsis is correlated with mortality and severity score. *Critical Care,* 17**,** 1-200.

SRISKANDAN, S. & COHEN, J. 1999. Gram-positive sepsis: mechanisms and differences from gram-negative sepsis. *Infectious disease clinics of North America,* 13**,** 397-412.

STARK, B., ANDREAE, F., MOSGOELLER, W., EDETSBERGER, M., GAUBITZER, E., KOEHLER, G. & PRASSL, R. 2008. Liposomal vasoactive intestinal peptide for lung application: protection

from proteolytic degradation. *European Journal of Pharmaceutics and Biopharmaceutics,* 70**,** 153-164.

STARK, B., DEBBAGE, P., ANDREAE, F., MOSGOELLER, W. & PRASSL, R. 2007. Association of vasoactive intestinal peptide with polymer-grafted liposomes: structural aspects for pulmonary delivery. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1768**,** 705-714.

STEELE-MORTIMER, O. 2008. The Salmonella-containing vacuole—moving with the times. *Current Opinion in Microbiology,* 11**,** 38-45.

STEELE-MORTIMER, O., MERESSE, S., GORVEL, J.-P., TOH, B.-H. & FINLAY, B. B. 1999. Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cellular Microbiology,* 1**,** 33-49.

STEELE-MORTIMER, O., BRUMELL, J. H., KNODLER, L. A., MÉRESSE, S., LOPEZ, A. & FINLAY, B. B. 2002. The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. *Cellular Microbiology,* 4**,** 43-54.

STENMARK, H. 2009. Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology,* 10**,** 513-525.

STEVENS, F. C. 1983. Calmodulin: an introduction. *Canadian journal of biochemistry and cell biology,* 61**,** 906-910.

STORKA, A., BURIAN, B., FÜHRLINGER, G., CLIVE, B., SUN, T., CREVENNA, R., GSUR, A., MOSGÖLLER, W. & WOLZT, M. 2013. VPAC1 receptor expression in peripheral blood mononuclear cells in a human endotoxemia model. *Journal of Translational Medicine,* 11**,** 1-6.

STOW, J. L., MANDERSON, A. P. & MURRAY, R. Z. 2006. SNAREing immunity: the role of SNAREs in the immune system. *Nature Reviews Immunology,* 6**,** 919-929.

STRANDBERG, K. L., RICHARDS, S. M. & GUNN, J. S. 2012. Cathelicidin antimicrobial peptide expression is not induced or required for bacterial clearance during salmonella enterica infection of human monocyte-derived macrophages. *Infection and Immunity,* 80**,** 3930-3938.

STREET, N. E. & MOSMANN, T. R. 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *The FASEB Journal,* 5**,** 171-177.

STUMHOFER, J. S., SILVER, J. S., LAURENCE, A., PORRETT, P. M., HARRIS, T. H., TURKA, L. A., ERNST, M., SARIS, C. J., O'SHEA, J. J. & HUNTER, C. A. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature Immunology,* 8**,** 1363-1371.

SUN, W., HONG, J., ZANG, Y. C., LIU, X. & ZHANG, J. Z. 2006. Altered expression of vasoactive intestinal peptide receptors in T lymphocytes and aberrant Th1 immunity in multiple sclerosis. *International Immunology,* 18**,** 1691-1700.

SUNDQUIST, M., JOHANSSON, C. & WICK, M. J. 2003. Dendritic cells as inducers of antimicrobial immunity in vivo. *Apmis,* 111**,** 715-724.

SUTTERWALA, F. S., MIJARES, L. A., LI, L., OGURA, Y., KAZMIERCZAK, B. I. & FLAVELL, R. A. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *The Journal of Experimental Medicine,* 204**,** 3235-3245.

SZEMA, A. M., HAMIDI, S. A., KOLLER, A. & MARTIN, D. W. 2011. Vasoactive Intestinal Peptide Knockout (VIP KO) mouse model of sulfite-sensitive asthma: up-regulation of novel lung carbonyl reductase. *BMC Immunology,* 12**,** 66.

SZETO, J., NAMOLOVAN, A., OSBORNE, S. E., COOMBES, B. K. & BRUMELL, J. H. 2009. Salmonella-containing vacuoles display centrifugal movement associated with cell-to-cell transfer in epithelial cells. *Infection and Immunity,* 77**,** 996-1007.

TAKEUCHI, O., HOSHINO, K., KAWAI, T., SANJO, H., TAKADA, H., OGAWA, T., TAKEDA, K. & AKIRA, S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity,* 11**,** 443-451.

TAM, M. A., RYDSTRÖM, A., SUNDQUIST, M. & WICK, M. J. 2008. Early cellular responses to Salmonella infection: dendritic cells, monocytes, and more. *Immunological Reviews,* 225**,** 140-162.

TANAKA, M., MIYOSHI, J., ISHIZAKI, H., TOGAWA, A., OHNISHI, K., ENDO, K., MATSUBARA, K., MIZOGUCHI, A., NAGANO, T. & SATO, M. 2001. Role of Rab3 GDP/GTP exchange protein in synaptic vesicle trafficking at the mouse neuromuscular junction. *Molecular Biology of the Cell,* 12**,** 1421-1430.

TAO, Q., REN, J. & LI, J. 2007. Vasoactive intestinal peptide inhibits adhesion molecule expression in activated human colon serosal fibroblasts by preventing NF-κB activation. *Journal of Surgical Research,* 140**,** 84-89.

TAPPING, R. I., AKASHI, S., MIYAKE, K., GODOWSKI, P. J. & TOBIAS, P. S. 2000. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. *The Journal of Immunology,* 165**,** 5780-5787.

TAUXE, R., KRUSE, H., HEDBERG, C., POTTER, M., MADDEN, J. & WACHSMUTH, K. 1997. Microbial hazards and emerging issues associated with produce: a preliminary report to the National

Advisory Committee on Microbiologic Criteria for Foods. *Journal of Food Protection®,* 60**,** 1400-1408.

TEMEROZO, J. R., JOAQUIM, R., REGIS, E. G., SAVINO, W. & BOU-HABIB, D. C. 2013. Macrophage resistance to HIV-1 infection is enhanced by the neuropeptides VIP and PACAP. *PLoS One* ,6 , e67701.

THIAGARAJAN, R., TEWOLDE, T., LI, Y., BECKER, P. L., RICH, M. M. & ENGISCH, K. L. 2004. Rab3A negatively regulates activity-dependent modulation of exocytosis in bovine adrenal chromaffin cells. *The Journal of Physiology,* 555**,** 439-457.

THOMPSON, M., KLIEWER, A., MAASS, D., BECKER, L., WHITE, D. J., BRYANT, D., ARTEAGA, G., HORTON, J. & GIROIR, B. P. 2000. Increased cardiomyocyte intracellular calcium during endotoxin-induced cardiac dysfunction in guinea pigs. *Pediatric Research,* 47**,** 669-676.

THORNDYKE, M. C., RIDDELL, J. H., THWAITES, D. T. & DIMALINE, R. 1989. Vasoactive intestinal polypeptide and its relatives: Biochemistry, distribution, and functions. *Biological Bulletin***,** 183-186.

TOBIAS, P., MATHISON, J., MINTZ, D., LEE, J., KRAVCHENKO, V., KATO, K., PUGIN, J. & ULEVITCH, R. 1992. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. *Am J Respir Cell Mol Biol,* 7**,** 239-245.

TOBIAS, P. S., SOLDAU, K. & ULEVITCH, R. J. 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *The Journal of experimental medicine,* 164**,** 777-793.

TORRES, K. C., DUTRA, W. O. & GOLLOB, K. J. 2004. Endogenous IL-4 and IFN-γ are essential for expression of Th2, but not Th1 cytokine message during the early differentiation of human CD4+ T helper cells. *Human Immunology,* 65**,** 1328-1335.

TRACEY, K. J., BEUTLER, B., LOWRY, S. F., MERRYWEATHER, J., WOLPE, S., MILSARK, I. W., HARIRI, R. J., FAHEY, T. J., ZENTELLA, A. & ALBERT, J. D. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science,* 234**,** 470-474.

TRACEY, M., KEVIN J & CERAMI, P. D., ANTHONY 1994. Tumor necrosis factor: A pleiotropic cytokine and therapuetic target. *Annual Review of Medicine,* 45**,** 491-503.

TRINCHIERI, G. 1994. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood,* 84**,** 4008-4027.

TSUBOI, T. & FUKUDA, M. 2006. Rab3A and Rab27A cooperatively regulate the docking step of dense-core vesicle exocytosis in PC12 cells. *Journal of Cell Science,* 119**,** 2196-2203.

ULEVITCH, R. J. & TOBIAS, P. S. 1994. Recognition of endotoxin by cells leading to transmembrane signaling. *Current Opinion in Immunology,* 6**,** 125-130.

ULLOA, L. & TRACEY, K. J. 2005. The 'cytokine profile': a code for sepsis. *Trends in Molecular Medicine,* 11**,** 56-63.

ULLRICH, O., REINSCH, S., URBÉ, S., ZERIAL, M. & PARTON, R. G. 1996. Rab11 regulates recycling through the pericentriolar recycling endosome. *The Journal of Cell Biology,* 135**,** 913-924.

URONEN-HANSSON, H., ALLEN, J., OSMAN, M., SQUIRES, G., KLEIN, N. & CALLARD, R. E. 2004. Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. *Immunology,* 111**,** 173-178.

USDIN, T., BONNER, T. & MEZEY, E. 1994. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology,* 135**,** 2662-2680.

VAN DE VEERDONK, F. L., STOECKMAN, A. K., WU, G., BOECKERMANN, A. N., AZAM, T., NETEA, M. G., JOOSTEN, L. A., VAN DER MEER, J. W., HAO, R. & KALABOKIS, V. 2012. IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proceedings of the National Academy of Sciences,* 109**,** 3001-3005.

VANNIER, E., MILLER, L. C. & DINARELLO, C. A. 1992. Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. *Proceedings of the National Academy of Sciences,* 89**,** 4076-4080.

VAROL, C., LANDSMAN, L., FOGG, D. K., GREENSHTEIN, L., GILDOR, B., MARGALIT, R., KALCHENKO, V., GEISSMANN, F. & JUNG, S. 2007. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *The Journal of Experimental Medicine,* 204**,** 171-180.

VAUDRY, D., GONZALEZ, B. J., BASILLE, M., YON, L., FOURNIER, A. & VAUDRY, H. 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological Reviews,* 52**,** 269-324.

VIGNALI, D. A. & KUCHROO, V. K. 2012. IL-12 family cytokines: immunological playmakers. *Nature Immunology,* 13**,** 722-728.

VIJAYAN, E., SAMSON, W., SAID, S. & MCCANN, S. 1979. Vasoactive Intestinal Peptide: Evidence for a Hypothalamic Site of Action to Release Growth Hormone, Luteinizing Hormone, and Prolactin in Conscious Ovariectomized Rats*. *Endocrinology,* 104**,** 53-57.

VOICE, J. K., DORSAM, G., LEE, H., KONG, Y. & GOETZL, E. J. 2001. Allergic diathesis in transgenic mice with constitutive T cell expression of inducible vasoactive intestinal peptide receptor. *The FASEB Journal,* 15**,** 2489-2496.

VON MOLLARD, G. F., STAHL, B., KHOKHLATCHEV, A., SÜDHOF, T. & JAHN, R. 1994. Rab3C is a synaptic vesicle protein that dissociates from synaptic vesicles after stimulation of exocytosis. *Journal of Biological Chemistry,* 269**,** 10971-10974.

VON ZASTROW, M. 2003. Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sciences,* 74**,** 217-224.

VOOGT, N., WANNET, W., NAGELKERKE, N. & HENKEN, A. 2002. Differences between national reference laboratories of the European community in their ability to serotype Salmonella species. *European Journal of Clinical Microbiology and Infectious Diseases,* 21**,** 204-208.

VREUGDENHIL, A. C., DENTENER, M. A., SNOEK, A. P., GREVE, J.-W. M. & BUURMAN, W. A. 1999. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *The Journal of Immunology,* 163**,** 2792-2798.

WALLACH, D., VARFOLOMEEV, E., MALININ, N., GOLTSEV, Y. V., KOVALENKO, A. & BOLDIN, M. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annual Review of Immunology,* 17**,** 331-367.

WANG, H., YANG, H., CZURA, C. J., SAMA, A. E. & TRACEY, K. J. 2001. HMGB1 as a late mediator of lethal systemic inflammation. *American Journal of Respiratory and Critical Care Medicine,* 164**,** 1768-1773.

WANG, J. E., DAHLE, M. K., MCDONALD, M., FOSTER, S. J., AASEN, A. O. & THIEMERMANN, C. 2003. Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock,* 20**,** 402-414.

WANG, L., LI, L., WANG, L., YANG, J., WANG, J., ZHOU, Z., ZHANG, H. & SONG, L. 2013. Two Rab GTPases, EsRab-1 and EsRab-3, involved in anti-bacterial response of Chinese mitten crab Eriocheir sinensis. *Fish & Shellfish Immunology,* 35**,** 1007-1015.

WANG, R., ZHANG, Y., LIU, S., LI, C., SUN, L., BAO, L., FENG, J. & LIU, Z. 2014. Analysis of 52 Rab GTPases from channel catfish and their involvement in immune responses after bacterial

infections. *Developmental & Comparative Immunology,* 45**,** 21-34.

WANG, Y. & CONLON, J. M. 1995. Purification and structural characterization of vasoactive intestinal polypeptide from the trout and bowfin. *General and Comparative Endocrinology,* 98**,** 94-101.

WARD, N. S., CASSERLY, B. & AYALA, A. 2008. The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. *Clinics in Chest Medicine,* 29**,** 617-625.

WEAVER, C. T., HARRINGTON, L. E., MANGAN, P. R., GAVRIELI, M. & MURPHY, K. M. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity,* 24**,** 677-688.

WEBER, T. J., SMALLWOOD, H. S., KATHMANN, L. E., MARKILLIE, L. M., SQUIER, T. C. & THRALL, B. D. 2006. Functional link between TNF biosynthesis and CaM-dependent activation of inducible nitric oxide synthase in RAW 264.7 macrophages. *American Journal of Physiology-Cell Physiology,* 290**,** C1512-C1520.

WEHRHAHN, J., KRAFT, R., HARTENECK, C. & HAUSCHILDT, S. 2010. Transient receptor potential melastatin 2 is required for lipopolysaccharide-induced cytokine production in human monocytes. *The Journal of Immunology,* 184**,** 2386-2393.

WENZEL, R. P., PINSKY, M. R., ULEVITCH, R. J. & YOUNG, L. 1996. Current understanding of sepsis. *Clinical Infectious Diseases***,** 407-412.

WERSHIL, B. K., TURCK, C. W., SREEDHARAN, S. P., YANG, J., AN, S., GALLI, S. J. & GOETZL, E. J. 1993. Variants of vasoactive intestinal peptide in mouse mast cells and rat basophilic leukemia cells. *Cellular Immunology,* 151**,** 369-378.

WICK, M. J. 2003. The role of dendritic cells in the immune response to Salmonella. *Immunology Letters,* 85**,** 99-102.

WILCKE, M., JOHANNES, L., GALLI, T., MAYAU, V., GOUD, B. & SALAMERO, J. 2000. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. *The Journal of Cell Biology,* 151**,** 1207-1220.

WILSON, E. H., WILLE-REECE, U., DZIERSZINSKI, F. & HUNTER, C. A. 2005. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. *Journal of Neuroimmunology,* 165**,** 63-74.

WONG, K.-F. & LUK, J. M. 2009. Endotoxin-neutralizing peptides as gram-negative sepsis therapeutics. *Protein and peptide Letters,* 16**,** 539-542.

WU, D., LEE, D. & SUNG, Y. K. 2011. Prospect of vasoactive intestinal peptide therapy for COPD/PAH and asthma: a review. *Respir Res,* 12**,** 45.

WURSTER, A. L., TANAKA, T. & GRUSBY, M. J. 2000. The biology of Stat4 and Stat6. *Oncogene,* 19**,** 2577-2584.

WYKES, M. 2003. Why do B cells produce CD40 ligand? *Immunology and cell biology,* 81**,** 328-331.

XIN, Z., JIANG, X., WANG, H.-Y., DENNY, T. N., DITTEL, B. N. & GANEA, D. 1997. Effect of vasoactive intestinal peptide (VIP) on cytokine production and expression of VIP receptors in thymocyte subsets. *Regulatory Peptides,* 72**,** 41-54.

XIU, F. & JESCHKE, M. G. 2013. Perturbed mononuclear phagocyte system in severely burned and septic patients. *Shock (Augusta, Ga.),* 40**,** 81-88.

XU, D., TRAJKOVIC, V., HUNTER, D., LEUNG, B. P., SCHULZ, K., GRACIE, J. A., MCINNES, I. B. & LIEW, F. Y. 2000. IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background. *European Journal of Immunology,* 30**,** 3147-3156.

YADAV, M., HUANG, M.-C. & GOETZL, E. J. 2011. VPAC1 (vasoactive intestinal peptide (VIP) receptor type 1) G protein-coupled receptor mediation of VIP enhancement of murine experimental colitis. *Cellular Immunology,* 267**,** 124-132.

YAGUPSKY, P. & NOLTE, F. 1990. Quantitative aspects of septicemia. *Clinical Microbiology Reviews,* 3**,** 269-279.

YAMAGUCHI, K., TANAKA, M., MIZOGUCHI, A., HIRATA, Y., ISHIZAKI, H., KANEKO, K., MIYOSHI, J. & TAKAI, Y. 2002. A GDP/GTP exchange protein for the Rab3 small G protein family up-regulates a postdocking step of synaptic exocytosis in central synapses. *Proceedings of the National Academy of Sciences,* 99**,** 14536-14541.

YANG, H., OCHANI, M., LI, J., QIANG, X., TANOVIC, M., HARRIS, H. E., SUSARLA, S. M., ULLOA, L., WANG, H. & DIRAIMO, R. 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proceedings of the National Academy of Sciences,* 101**,** 296-301.

YAZDI, A. S., GUARDA, G., RITEAU, N., DREXLER, S. K., TARDIVEL, A., COUILLIN, I. & TSCHOPP, J. 2010. Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1α and IL-1β. *Proceedings of the National Academy of Sciences,* 107**,** 19449-19454.

YOON, H., MCDERMOTT, J. E., PORWOLLIK, S., MCCLELLAND, M. & HEFFRON, F. 2009. Coordinated regulation of virulence during

systemic infection of Salmonella enterica serovar Typhimurium. *PLoS Pathog*. 2, e1000306.

YU, R.-J., TAM, N.-L., GAO, Y., ZENG, Z.-H., ZHOU, T.-H. & HONG, A. 2007. A novel recombinant, VPAC2-selective agonist enhancing insulin release and glucose disposal. *Acta Pharmacologica Sinica,* 28**,** 526-533.

YUNES, R., TOMES, C., MICHAUT, M., DE BLAS, G., RODRIGUEZ, F., REGAZZI, R. & MAYORGA, L. 2002. Rab3A and calmodulin regulate acrosomal exocytosis by mechanisms that do not require a direct interaction. *FEBS Letters,* 525**,** 126-130.

ZAMIR, O., HASSELGREN, P.-O., HIGASHIGUCHI, T., FREDERICK, J. A. & FISCHER, J. E. 1992. Effect of sepsis or cytokine administration on release of gut peptides. *The American Journal of Surgery,* 163**,** 181-185.

ZERIAL, M. & MCBRIDE, H. 2001. Rab proteins as membrane organizers. *Nature Reviews Molecular Cell Biology,* 2**,** 107-117.

ZHANG, K., WANG, P., HUANG, S., WANG, X., LI, T., JIN, Y., HEHIR, M. & XU, C. 2014. Different mechanism of LPS-induced calcium increase in human lung epithelial cell and microvascular endothelial cell: a cell culture study in a model for ARDS. *Molecular Biology Reports,* 41**,** 4253-4259.

ZHANG, L.-T., YAO, Y.-M., LU, J.-Q., YAN, X.-J., YU, Y. & SHENG, Z.-Y. 2008. Recombinant bactericidal/permeability-increasing protein inhibits endotoxin-induced high-mobility group box 1 protein gene expression in sepsis. *Shock,* 29**,** 278-284.

ZHANG, R., ZHAO, M., JI, H.-J., YUAN, Y.-H. & CHEN, N.-H. 2013. Study on the Dynamic Changes in Synaptic Vesicle-Associated Protein and Axonal Transport Protein Combined with LPS Neuroinflammation Model. *ISRN Neurology,* 2013.

ZHANG, X., GUO, L., COLLAGE, R. D., STRIPAY, J. L., TSUNG, A., LEE, J. S. & ROSENGART, M. R. 2011. Calcium/calmodulin-dependent protein kinase (CaMK) Iα mediates the macrophage inflammatory response to sepsis. *Journal of Leukocyte Biology,* 90**,** 249-261.

ZHANG, Y.-C., ZUO, W.-Q., RONG, Q.-F., TENG, G.-L. & ZHANG, Y.-M. 2010. Glucocorticoid receptor expression on acute lung injury induced by endotoxin in rats. *World Journal of Emergency Medicine,* 1**,** 65.

ZHANG, Y., YANG, L., TANG, D. & ZHANG, Y. 2006. [Protective effects of vasoactive intestinal peptide on intestinal lesions induced by endotoxic shock in rat]. *Zhonghua er ke za zhi. Chinese Journal of Pediatrics,* 44**,** 369-373.

ZHAO, C., TAN, Y., WONG, W., SEM, X., ZHANG, H., HAN, H., ONG, S., WONG, K., YEAP, W. & SZE, S. 2010. The CD14+/lowCD16+ monocyte subset is more

susceptible to spontaneous and oxidant-induced apoptosis than the CD14+ CD16− subset. *Cell Death & Disease,* 1**,** e95.

ZHAO, C., WOOD, M. W., GALYOV, E. E., HÖPKEN, U. E., LIPP, M., BODMER, H. C., TOUGH, D. F. & CARTER, R. W. 2006. Salmonella typhimurium infection triggers dendritic cells and macrophages to adopt distinct migration patterns in vivo. *European Journal of Immunology,* 36**,** 2939-2950.

ZHAO, Y., YANG, J., SHI, J., GONG, Y.-N., LU, Q., XU, H., LIU, L. & SHAO, F. 2011. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature,* 477**,** 596-600.

ZHOU, H., HUANG, J. & MURTHY, K. S. 2006a. Molecular cloning and functional expression of a VIP-specific receptor. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 291**,** G728-G734.

ZHOU, X., MANTIS, N., ZHANG, X. R., POTOKA, D. A., WATKINS, S. C. & FORD, H. R. 2000. Salmonella typhimurium Induces Apoptosis in Human Monocyte-Derived Macrophages. *Microbiology and Immunology,* 44**,** 987-995.

ZHOU, X., YANG, W. & LI, J. 2006b. Ca2+-and protein kinase C-dependent signaling pathway for nuclear factor-κB activation, inducible nitric-oxide synthase expression, and tumor necrosis factor-α production in lipopolysaccharide-stimulated rat peritoneal macrophages. *Journal of Biological Chemistry,* 281**,** 31337-31347.

ZHU, J. & PAUL, W. E. 2008. CD4 T cells: fates, functions, and faults. *Blood,* 112**,** 1557-1569.

ZUO, W., ZHANG, Y., GONG, X. & ZHANG, Y. 2010. [Effects of vasoactive intestinal peptide on Toll-like receptor (TLR) 2 mRNA and TLR4 mRNA expression on acute lung injury induced by lipopolysaccharide in rat]. *Zhonghua er ke za zhi. Chinese Journal of Pediatrics,* 48**,** 19-23.