The role of the immune response in the effectiveness of antibiotic treatment for antibiotic susceptible and antibiotic resistant bacteria.

by

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Abstract

The increasing spread of antimicrobial resistant bacteria and the decline in the development of novel antibiotics have incited exploration of other avenues for antimicrobial therapy. One option is the use of antibiotics that enhance beneficial aspects of the host's defences to infection. This study explores the influence of antibiotics on the innate immune response to bacteria. The aims were to investigate antibiotic effects on bacterial viability, innate immune cells (neutrophils and macrophages) in response to bacteria and interactions between bacteria and the host. Five exemplar antibiotics; ciprofloxacin, tetracycline, ceftriaxone, azithromycin and streptomycin at maximum serum concentration (C_{max}) and minimum inhibitory concentrations (MIC) were tested. These five antibiotics were chosen as they are commonly used to treat infections and represent different classes of drug. *Salmonella* Typhimurium was chosen as the model pathogen as it is an intracellular organism, and as such the effect of antibiotics on entry and survival could be assessed. SL1344 was chosen because it is the wild type pathogenic *S*. Typhimurium strain and is routinely used in tissue culture infection assays. Following incubation of *S.* Typhimurium SL1344 with C_{max} concentrations of ceftriaxone bacterial viability was undetectable, ciprofloxacin reduced bacteria, while tetracycline, azithromycin and streptomycin did not alter bacteria viability. When *S.* Typhimurium was incubated with antibiotic treated neutrophils, there was reduced bacteria viability for ceftriaxone and ciprofloxacin. Neutrophils treated with ciprofloxacin had reduced ability to phagocytose *Escherichia coli*, while oxidative burst was increased following exposure to ceftriaxone.

Adhesion of *S.* Typhimurium SL1344 to J774 macrophage monolayers was increased when the macrophages were pre-exposed to C_{max} and MIC of ciprofloxacin and ceftriaxone, but only C_{max} concentrations of azithromycin and streptomycin. Bacterial adhesion was not altered when macrophages were pre-exposed to tetracycline. Invasion of the less invasive SL1344 *tolC::aph* was greater in macrophages exposed to antibiotics. Unlike the murine macrophages, antibiotics had no effect when *S.* Typhimurium was used to infect human cells (THP-1 and monocyte derived macrophages).

Expression of IL-1β and TNFα mRNA was greater in SL1344 infected macrophages which had been pre-exposed to ciprofloxacin or ceftriaxone, than in macrophages exposed to antibiotics alone or SL1344 alone.

In conclusion, it was found that clinical relevant concentrations of certain antibiotics enhance the response of immune cells and their interaction with bacteria, by increasing phagocytosis and killing in neutrophils, increasing bacterial adhesion to macrophages and increased cytokine production. These immunomodulatory potentials of antibiotics can be harnessed and exploited for broader therapeutic use.

for Dad and Mum

Acknowledgement

Table of contents

List of figures

List of Tables

List of Abbreviations

APC	Antigen presenting cells
AZI	Azithromycin
BCR	B cell receptor
BMDM	Bone marrow derived macrophages
CFU	Colony forming unit
CGD	Chronic granulomatous disease
CIP	Ciprofloxacin
C_{max}	Maximum serum concentration
CRO	Ceftriaxone
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPB	Diffuse pan bronchiolitis
DPBS	Dulbecco's phosphate buffered saline
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
FACS	Fluorescent activated cell sorting
FAE	Follicle-associated epithelium
FcR	Fragment crystallisable receptors
FITC	Fluorescein isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
FSC	Forward scatter
GM-CSF	Granulocyte macrophage –colony stimulating factor
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus

ICAM	Intracellular adhesion molecule
IDR	Immune defence regulator peptides
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
LB	Luria Broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
MBL	Mannose-binding lectin
M-CSF	Macrophage colony stimulating factor
MCWE	*Mycobacterium phlei* cell wall extract
MDM	Monocyte derived macrophages
MDR-TB	Multi-drug resistance tuberculosis
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MPS	Mononuclear phagocyte system
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acids
NET	Neutrophil extracellular trap

NF-κβ	Nuclear factor kappa beta
NK	Natural killer
NLR	NOD like receptors
NO	Nitric oxide
NTS	Non-typhoidal salmonellosis
PABA	Para-aminobenzoic acid
PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PKR	Protein kinase R
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear neutrophils
PP	Peyer's patch
PRR	Pathogen recognition receptors
RLR	RIG like receptors
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCV	*Salmonella* containing vacuole
SPI	*Salmonella* pathogenicity Island
SSC	Side scatter
SSI	Surgical site injection
TET	Tetracycline
TLR	Toll like receptors
TNFα	Tumour necrosis factor alpha

TTSS	Type three secretion system
UTI	Urinary tract infection
VCAM-1	Vascular cell adhesion molecule-1
VRE	Vancomycin-resistant *Enterococcus*
XDR-TB	Extensively-drug resistant tuberculosis

CHAPTER ONE

1 Introduction

1.1 Immune system overview

The immune system comprises a vast network of mechanisms that work hand in hand to protect an organism from invading pathogens. Each arm of the immune system contributes in recognition and elimination of pathogens (Iwasaki and Medzhitov, 2010). In order to achieve this, the host depends on the immune system's ability to distinguish between host cells and invading pathogens by virtue of unique structural patterns, allowing the host to clear pathogens without causing damage to self-tissues. Cells of the immune system originate from hematopoietic stem cells found in the bone marrow (Chaplin, 2006). Haematopoiesis occurs at different anatomical locations during the developmental stages of an organism. Changes in anatomical sites of haematopoiesis indicate the different functional status of the stem cells and the changing needs of the developing organism. Haematopoiesis during the embryonic stage occurs in two stages: primitive and definitive haematopoiesis. Primitive haematopoiesis occurs in the yolk sac, aorta-gonad-mesonephros and placenta, before the development of the fetal liver. The yolk sac is the earliest site where macrophages, erythrocytes and megakaryotes are found. Definitive haematopoiesis occurs in the embryo after the development of the liver. At 14-20 weeks post gestation until post natal stages, haematopoiesis occurs in the liver and spleen, while haematopoiesis occurs exclusively in the bone marrow in adults (Mosaad, 2014). The stem cells make their way through the blood and lymph, forming microstructures in lymphoid organs, and eventually permeate body tissue (Alberts et al., 2002). This circulation through the blood and permeation of tissues is the crux of an effective immune response against invading pathogens.

Recognition of the distinctively unique structural patterns on pathogens, employs two host mechanisms; 1) The innate immune system, an evolutionarily conserved system that recognises a wide range of pathogen-associated molecular patterns (PAMP) within hours of infection (Janeway and Medzhitov, 2002). This is achieved using pattern recognition receptors (PRR) such as Nod-like receptors (Inohara, 2001) and Toll-like receptors (Hoffmann, 1999) and provides a rapid 'first aid' response against invading microbes. 2) The adaptive immune system which constitutes cells with exquisite specificity for individual microbial molecular structures that frequently undergo mutation to change the recognised structures to avoid being detected by the immune system. The primary adaptive immune response is triggered after the innate immune system but produces a long lived response (years) and immune memory in the event of a re-infection or a second challenge. Although classified separately, activities of these two systems overlap and contribute effectively in combating infections and was described by Chaplin (2006) as "complementary and cooperating".

The hallmark of the immune response is immunological recognition; the ability to distinguish between self and non-self (Medzhitov and Janeway, 2000). Without this, cells of the immune system could react against host own tissues leading to development of autoimmune diseases. This function is expressed in both the innate and adaptive immune system and is discussed subsequently.

1.1.1 Innate immune response

Pathogens gain entry into the host through various routes. The innate immune system sits at the frontline of a host defence system. It encompasses physical barriers; epithelial cells of the skin, mucosal linings of the respiratory tract, gastrointestinal tract and upper respiratory tract, and the epithelial cilia that ensure

that inhaled particles are swept away from the lungs. Beneath the epithelial surfaces lie phagocytes that engulf and digest invading pathogens. Although described as primitive and limited, studies have illustrated that in the absence of an adaptive immune system, these phylogenetically conserved mechanisms offer protection against fatal pathogens (Melotto et al., 2006, Kunze et al., 2004). The mammalian innate immune system not only recognises pathogens, but primes and modulates the clonal expansion of antigen-responsive cells by the adaptive immune system (Medzhitov et al., 1997, Gururajan et al., 2007).

1.1.1.1 Activation of Innate Immunity.

The cellular component of the innate immune system (basophils, eosinophils, macrophages/monocytes, neutrophils, natural killer (NK) cells and dendritic cells) depend on a number of soluble factors and receptors for the generation of a non-specific immune response. Using an activation program, these cells are able to sense the presence of an infection, damage to host cells and tissues.

1.1.1.1.1 Activation of innate immunity by Pattern Recognition Receptors (PRRs)

The innate immune system recognises conserved molecular patterns, Pathogen-associated molecular patterns (PAMP) shared by large groups of pathogens, using PRRs. PAMPs include peptidoglycans and lipoteichoic acid (LTA) (in Gram positive bacteria), lipopolysaccharide (in Gram negative bacteria), mannans, glucans, double stranded RNA and bacterial DNA (Figure 1.1). PRRs have been classified into 1) humoral proteins, 2) endocytic receptors and 3) signalling receptors (Franchi, 2006, Declue et al., 2012). Humoral proteins include mannose binding lectins, collectins and C-reactive proteins. These proteins identify the invading pathogen, bind to, and

opsonise them. Furthermore, they activate the complement system, which neutralises opsonised pathogens and eventually clears it from the hosts' system. Endocytic receptors assist in the degradation of captured pathogens or damaged cells by facilitating their internalisation and transportation to the lysosomal compartment. They include scavenger receptors and c-type lectins (Sancho and Reis e Sousa, 2013). Both the humoral proteins and the endocytic receptors have important functions activating antigen presentation, antigens are internalised into endosomes, degraded to peptides and presented on major histocompatibility complex II to T cells, subsequently inducing T cell responses (Malhotra et al., 2009, Burgdorf and Kurts, 2008). The signalling receptors are the principal sensors of pathogens. They sense cell death and signal the up-regulation of immune response genes necessary for induction of effector cells and adaptive immune responses. Signalling receptors identified are Toll-like receptors (TLR), Nod-like receptors (NLR) and the retinoic acid inducible gene-I (RIG-I)-like receptors (RLR) (Creagh and O'Neill, 2006).

Figure 1.1 Pathogen associated molecular patterns.
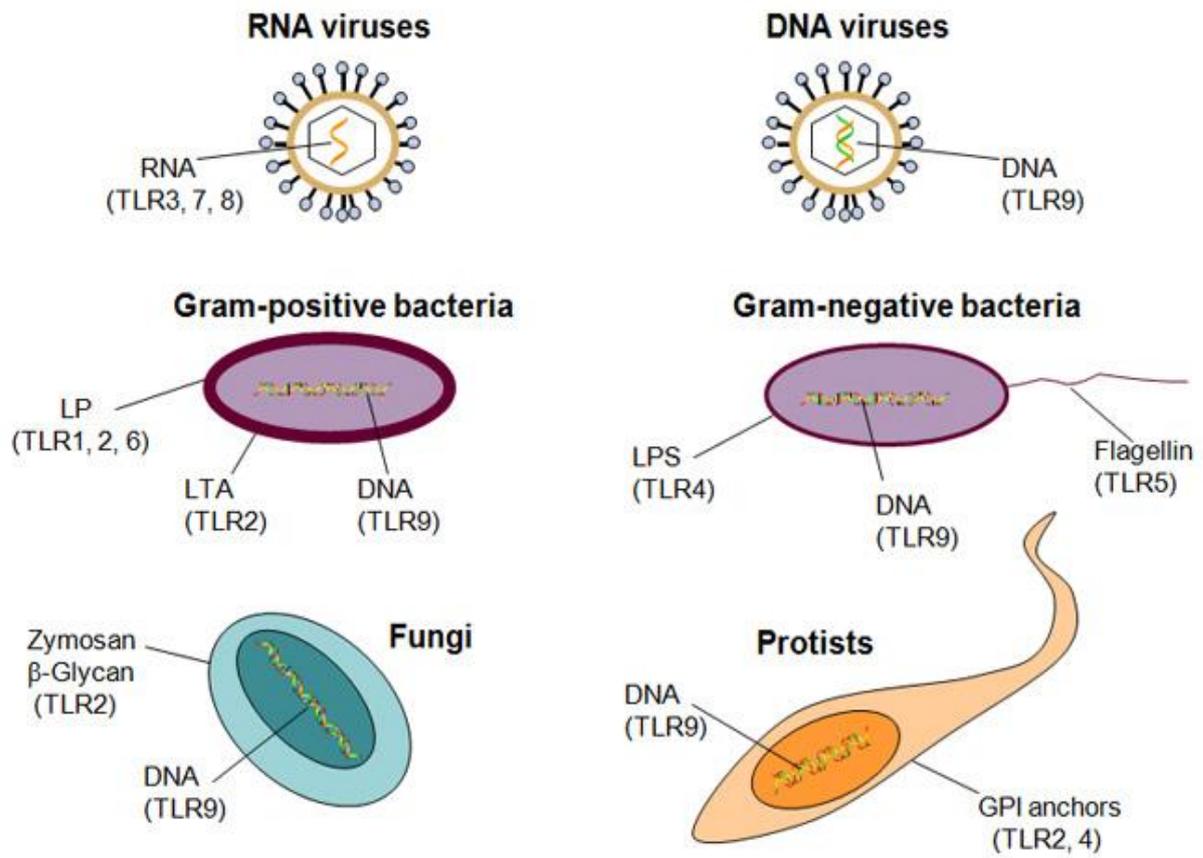
Figure 1.1 The unique pathogen associated molecular patterns that have been identified in different pathogens. For instance, RNA and DNA in viruses, lipoproteins (LP), lipoteichoic acid (LTA) and lipopolysaccharide (LPS) in Gram positive bacteria, LPS and flagella in Gram negative bacteria, zymosan in fungi and glycosylphosphatidylinositol (GPI) surface proteins in protists (Christmas, 2010).

TLR control nuclear factor kappa beta (NF-κβ) signalling activity and influence expression of cell surface molecules and inflammatory cytokines. These molecules are germline encoded, found on a number of effector cells and are able to differentiate between self and non-self, making them less harmful to host cells. NLRs are primarily expressed in lymphocytes and antigen presenting cells such as macrophages and dendritic cells.

Toll like receptors are the best characterized of the pathogen recognition receptors. They are typically expressed on the surface of immune cells such as macrophages,

dendritic cells and neutrophils (Hancock et al., 2012, Hayashi et al., 2003) In humans, there are 10 TLR members designated TLRs 1-10 while the TLR family consists of 13 members (Lee et al., 2014). The NLR family in humans consists of 22 proteins and 33 proteins in mice. As in TLR signalling, NLR signalling is activated on recognition of PAMPs. Three major targets of NLR signalling after PAMP recognition are NF-κβ, mitogen activated protein kinase (MAPK) and caspase-1 (Chen et al., 2009).

Sansonetti (2006) argues that TLRs and NLRs not only respond to pathogenic molecular pattern but also respond to endogenous molecules (alarm signals) produced by damaged or injured cells and elicit an immune response (Sansonetti, 2006). In other words, it could also mean that PAMPs and these endogenous alarm signals both share some commonalities and are ancient (Matzinger, 2007). It should be noted that expression of signalling receptors is not confined to cells of the immune system alone. Low expression of TLR2 and 4 has been reported in intestinal epithelial cells (Cario and Podolsky, 2000).

1.1.1.1.2   Activation of innate immunity by cytokines.

Cytokines are soluble low molecular weight proteins that are derived from many tissue sources (Janeway and Medzhitov, 2002). They function as chemical messengers where they relay information between cells, mediate effector cell differentiation and modulate immunological responses. The main cytokines of the innate immune system include interferon (IFN)-γ (secreted by NK cells) which activates macrophages, interleukin (IL)-15 and IL-12 (produced by dendritic cells and macrophages) which activates and regulates the proliferation of NK cells and IFN-γ (secreted by injured non immune cells) which activates both dendritic and NK cells

(Zhang et al., 2008, Harizi, 2013). Cells of the immune system have cytokine receptors with which they respond to these cytokines.

1.1.1.2 Effector cells of the Innate Immune system.

1.1.1.2.1 Macrophages

Macrophages are derived from monocytes that migrate out of the circulation and undergo proliferation and differentiation in tissue. They are found at all mucosal surfaces and in specialised organs e.g. the lungs (alveolar macrophages), liver (Kuffer cells), bone (osteoblasts), central nervous system (microglia) and synovial membrane (type A synoviocytes). PPRs and fragment crystallisable receptors (FcRs) on antibody trigger the activation of macrophages, and they have a vigorous response to the presence of the cytokines IFN-γ leading to their activation.

In the 1980s, van Furt and colleagues described the concept of mononuclear phagocyte system (MPS). MPS can be described as a group of cells that share a common bone marrow progenitor that differentiates into blood monocytes and gives rise to dendritic cells and tissue resident macrophages with the body (Hume, 2008). Geissmann et al., proposed two distinct murine monocyte subset based on their expression of Ly6C and CX3CR1 (Geissmann et al., 2003). Ly6C^{high} monocytes express intermediate levels of chemokine receptors CX3CR1, CCR2 and the adhesion molecule CD62L (L-selectin). Ly6C^{high} monocytes are referred to as inflammatory monocytes because they migrate to sites of inflammation, produce pro-inflammatory cytokines and cause tissue damage as seen in various disease models (Serbina and Pamer, 2006, Getts et al., 2008). Ly6C^{low} monocytes are characterised by high levels of CX3CR1 and CD43, but no expression of CCR2 and CD62L. The Ly6C^{low} monocytes are referred to as tissue resident macrophages because they

were found both in resting and inflamed tissues (Geissmann et al., 2003). It is postulated that Ly6C^{low} monocytes scavenge the vascular system for dead cells, and lipid and blood-borne pathogens, hence they are said to exhibit patrolling behaviour (Auffray et al., 2007).

Based on phenotype, morphology and cell surface molecule expression, circulating human blood monocytes are classified into CD14^{++}CD16^{-}, CD14^{+} CD16^{++} or CD14^{+}CD16^{-} monocytes (Ancuta et al., 2000). The CD14^{++}CD16^{-}, are the most prevalent subset and are referred to as the classical monocytes, while the CD14^{+} CD16^{++} and CD14^{+}CD16^{-} are referred to as the non-classical monocytes. The CD14^{++}CD16^{-} subset are similar to the mouse Ly6C^{high} monocytes in their expression of CCR2^{high}CX$_3$CR1^{low} chemokine. The non-classical CD14^{+} CD16^{++} and CD14^{+}CD16^{-} have similar expression of CX$_3$CR1^{high}CCR2^{low} as Ly6C^{low} murine macrophages which are suggested to be pro-inflammatory (Geissmann et al., 2003). Ingersoll and colleagues have shown close relationship between the classical human monocytes and the mouse Ly6C^{high} in gene expression profiles where 130 genes are conserved between the two subsets (Ingersoll et al., 2010).

Macrophages have been classified as either M1 or M2 macrophages, the M1 subsets being anti-inflammatory, while the M2 subset is tissue resident macrophages. Furthermore, depending on which cytokines induces their activation, the M2 class is sub classified into M2a (induced by IL-13 and IL-4), M2b (induced by agonists of TLRs, IL-1 and immune complexes) and M2c induced by glucocorticoid hormones and IL-10 (Mantovani et al., 2004). While the M1 class are microbicidal and inflammatory, the M2 class are poor microbicides but have immunomodulatory function. In other words, these findings suggest that macrophages could either be pro-inflammatory or anti-inflammatory in function. According to Porcheray et al,

(2005) activation of macrophages by bacterial infection is "plastic, rapid and fully reversible". In essence, macrophages participate in mounting inflammatory responses and also aid in the dissolution of these responses (Porcheray et al., 2005).

More recently, perspectives that challenge the concept that monocytes migrate out of circulation show that resident macrophages differentiate from foetal precursors. Yona et al, 2013 reported that resident macrophages were derived from the yolk sac and proliferate slowly in situ (Yona et al., 2013). However, it was shown that for certain tissues such as gut and skin that seeding from the blood may be relevant (Bain et al., 2014). Finally, transcriptome analysis showed that designating macrophages as resident or inflammatory may be misleading and that cells form a spectrum of types based on microenvironment (Hume and Freeman, 2014).

Macrophages respond to the presence of bacterial infection by up-regulating genes responsible for M1 polarisation. It was hypothesised that there is a common transcriptional activation programme that regulates the response of host cells exposed to various pathogens and that these common responses are regulated in the innate immune system (Jenner and Young, 2002). In support of this hypothesis, Nau showed that these common activation programs regulate the response of human monocyte derived macrophages to a broad range of bacteria (Nau et al., 2002). These genes encode the production of cytokines, cytokine receptors, chemokines and chemokine receptors. In addition, they also encode two enzymes involved in the microbicidal activities of macrophages via nitric oxide (NO) synthetase 2 and indoleamine-pyrole-2,3-dioxygenase as well as two co-stimulatory molecules CD80 and CD86 (Jenner and Young, 2002).

M1 activated macrophages protect the host in the event of acute infections and provide resistance to infection. For example *Listeria monocytogenes,* which causes disease in pregnant women and immunocompromised patients, was shown to induce M1 activated macrophages to kill these bacteria *in vivo* and *in vitro* as well as deterring bacterial phagosomes from escaping into the host system (Shaughnessy, 2007). Mice deficient in TNFα and IFN-γ and the various receptors for these cytokines when challenged with these bacteria succumb to infection, as they have severely impaired ability to clear the infection. These two cytokines are "canonical markers of M1 macrophage activation" (Pfeffer et al., 1993).

On the other hand, M2 responses are associated with chronic infections. They play a decisive role in the fate of chronic mycobacterial infections. Most tumour associated macrophages have phenotypic similarities to M2 macrophage (Saccani et al., 2006). Although macrophages control and resist the development of an acute infection, prolonged activation is dangerous to the host. In such cases there is a systemic immune inflammatory response, coupled with immune dysregulation which damages tissues and may lead to multiple organ failure (O'Reilly et al., 1999). Generally, M2 macrophages are tilted towards Th2 reactions. They are involved in the encapsulation and killing of pathogen, progression of tumours, repair and re-modelling of tissues (Wynn, 2004) as well as immuno-regulation (Gordon, 2003).

1.1.1.2.2   Neutrophils

Polymorphonuclear neutrophils, (PMN) are the most abundant of the circulating white blood cells. They are rapidly produced by, and respond quickly to the presence of invading pathogens. They have a life span of about 5.4 days in circulation (Pillay et

al., 2010) and have a rapid turnover of 50-340 x 10^7 cell/kg/day equating to approximately 0.4-3 x 10^{11} cell/day in a 75-kg individual (Kobayashi, 2009).

The rapid production, circulation and recruitment of neutrophils to tissues are highly important in innate immunity. The migration of neutrophils from the site of production to the extravascular milieu depends on signalling from soluble mediators and contact with the endothelial lining of capillary venules. L-selectin is a class of C–type lectin glycoprotein which is expressed on neutrophils and allows them adhere to, and roll along endothelial surfaces through low affinity interaction (Lawrence, 1991, Cummings and McEver, 2008). In the presence of inflammatory mediators activated neutrophils switch to high affinity adherence via β-integrins and intracellular adhesion molecules 1 and 2 (ICAM) 1 and 2. Once attached to these surfaces, neutrophils migrate through the endothelium into tissues and sites of infection in a process facilitated by neutrophil surface molecules such as CD31, CD44, CD47 and CD54 (Khan et al., 2004, Cooper et al., 1995). Localisation of these cells to sites of infection is a key factor in innate immunity. This neutrophil localisation to infected tissue is known as chemotaxis. CXCL8 has been listed as one of the most potent chemoattractants in neutrophil chemotaxis (De Larco, 2004). In addition, leukotrienes and complement component C5a are all potent neutrophil chemoattractants (Ehrengruber et al., 1995). In addition to directing PMN to sites of infection, chemoattractants also prime the functionality of these cells. Since these neutrophils are antimicrobial in function and produce antimicrobial cytotoxic molecules, activities of these cells have been linked to inflammatory diseases as well as tissue injury (Edwards and Hallett, 1997).

Antimicrobial activity of Neutrophils

The killing of pathogens by neutrophils occurs through a series of organised processes, resulting in the production of reactive oxygen species (ROS) as well fortification of the phagosome with cytotoxic molecules such as lysosomal proteases, defensins and myeloperoxidase. The ROS are derived from nicotinamide adenine dinucleotide phosphate (NADPH), an enzyme complex composed of seven proteins found in the cytosol (Quinn et al., 2006). NADPH catalyses the formation of superoxide which is further reduced to hydrogen peroxide. However, oxygen and hydrogen peroxide are weak microbicides. Maturation of the phagosome leads to the accumulation of myeloperoxidase within the phagosome. Myeloperoxidase catalyses the reaction of hydrogen peroxide and chloride to produce hypochlorous acid (HOCl), a bactericidal compound. Deficiencies in these oxygen species results in Chronic Granulomatous Disorder (CGD) with individuals suffering from recurrent infections due to the insufficient ROS (Quinn and Gauss, 2004). Although NADPH and hypochlorous acid are the key antimicrobial agents of neutrophils, (Rosen and Klebannof, 1979) demonstrated that deficiency of these factors does not correlate with morbidity during infections. Comparing neutrophils from MPO deficient and MPO competent individuals, show that neutrophils from MPO deficient individuals retain antimicrobial activity against microbes, but at a slower rate than in competent individuals. These findings suggest that the antimicrobial activities of neutrophils are multi-factorial.

In addition to the phagocytic process, neutrophils have been shown to kill extracellularly. According to Tobias *et al.,* when neutrophils are activated, they release web like structures consisting of chromatin and extracellular protein that capture and kill pathogens extracellularly. These structures are called Neutrophil

Extracellular Traps (NET) (Tobias et al., 2007). As the name suggest, this structure traps, disarms and destroys invading pathogens by exposure to high concentrations of antimicrobial effectors such as histones, enzymes e.g. MPO and neutrophil elastase, and proteins e.g. cathepsins and lactoferrin (Wang et al., 2009). NET trap released in response to bacteria is time and NADPH oxidase dependent (Riyapa et al., 2012), Because histones can disintegrate pathogen cell wall membranes, compromising pathogen viability (Mendez-Samperio, 2010), and NET associated proteases e.g. neutrophil elastase can inactivate and kill pathogens by cleaving their virulence factors (Brinkmann et al., 2004) NET are thought to be an effective antimicrobial mechanism. However, bacterial resistance to NET is reported in cystic fibrosis strains of *S. pneumoniae* (Beiter et al., 2006) and *P. aeruginosa* (Young et al., 2011). One of the proposed mechanisms for evasion of NET in *S. pnuemoniae* is through secretion of endonucleases that cleave DNA (Beiter et al., 2006).

1.1.1.2.4   Complement system.

The complement system consists of a tightly regulated cascade of soluble proteins (zymogens) in the blood that aid in the killing of bacteria by antibodies. Their role in host defence and inflammation is indispensable. They are a major factor in the opsonisation of bacteria by immune effector cells as well as in cell lysis. The action of complement is achieved through a plethora of responses that include chemotaxis of granulocytes and apoptosis of the target cell. There are three systems involved in the activation of complement; the alternative, classical and lectin pathways (Sarma and Ward, 2011). Proteins and lipids on surface of bacteria trigger the alternative pathway (Wu et al., 2009). Complement factor C3 is hydrolysed to C3b which then binds to bacteria. Factor B binds to the hydrolysed C3b, and then cleaved by factor D

forming the unstable C3bBb convertase. C3bBb is stabilised by properdin, a plasma protein secreted by neutrophils, macrophages and T cells (Flierl, 2008).

The lectin pathway is initiated when Mannose-binding lectin (MBL) binds to carbohydrate molecules on the surface of invading bacteria. MBL forms complexes with MBL-associated proteins (MASP) with subsets 1, 2 and 3 binding to pathogens in the lectin pathway (Kemper and Atkinson, 2007). Binding to pathogens activates MASP2 which cleaves complement factor C4 to C4a and C4b. C4b is then attached to the surface of bacteria which in turn induces the attachment of complement factor C2. MASP2 again cleaves C2 to C2a and C2b. C4b and C2a together form the lectin pathway C3 convertase, C4bC2a (Figure 1.2)

The classical pathway is activated upon antibody binding to bacteria. The C1 complement factor is multimeric, and consists of three molecules C1q, r and s. C1q binds to the Fc portion of antibody IgG or IgM leading to the activation of C1r and C1s. C1s cleave both C4 and C2 to form the classical pathway C3 convertase C4bC2a (Sarma and Ward, 2011). From this point the pathways converge as C3 is cleaved by the C3 convertases releasing C3a and C3b. C3a is an opsonin that helps in phagocytosis of bacteria, and further amplification of complement activation. C3b binds to the C3 convertases to form C4bC2aC3b and C3bBbC3b, which are C5 convertases, that cleave C5 to C5a and C5b. C5b binds to C6, C7, C8 and multiple C9 molecules resulting in formation of a membrane attack complex (MAC). The MAC forms pores in the invading pathogen, and inserts into their cells, resulting in the lysis of pathogenic cells (Figure 1.2) (Kondos et al., 2010).

Figure 1.2 Diagrammatic summary of the complement system
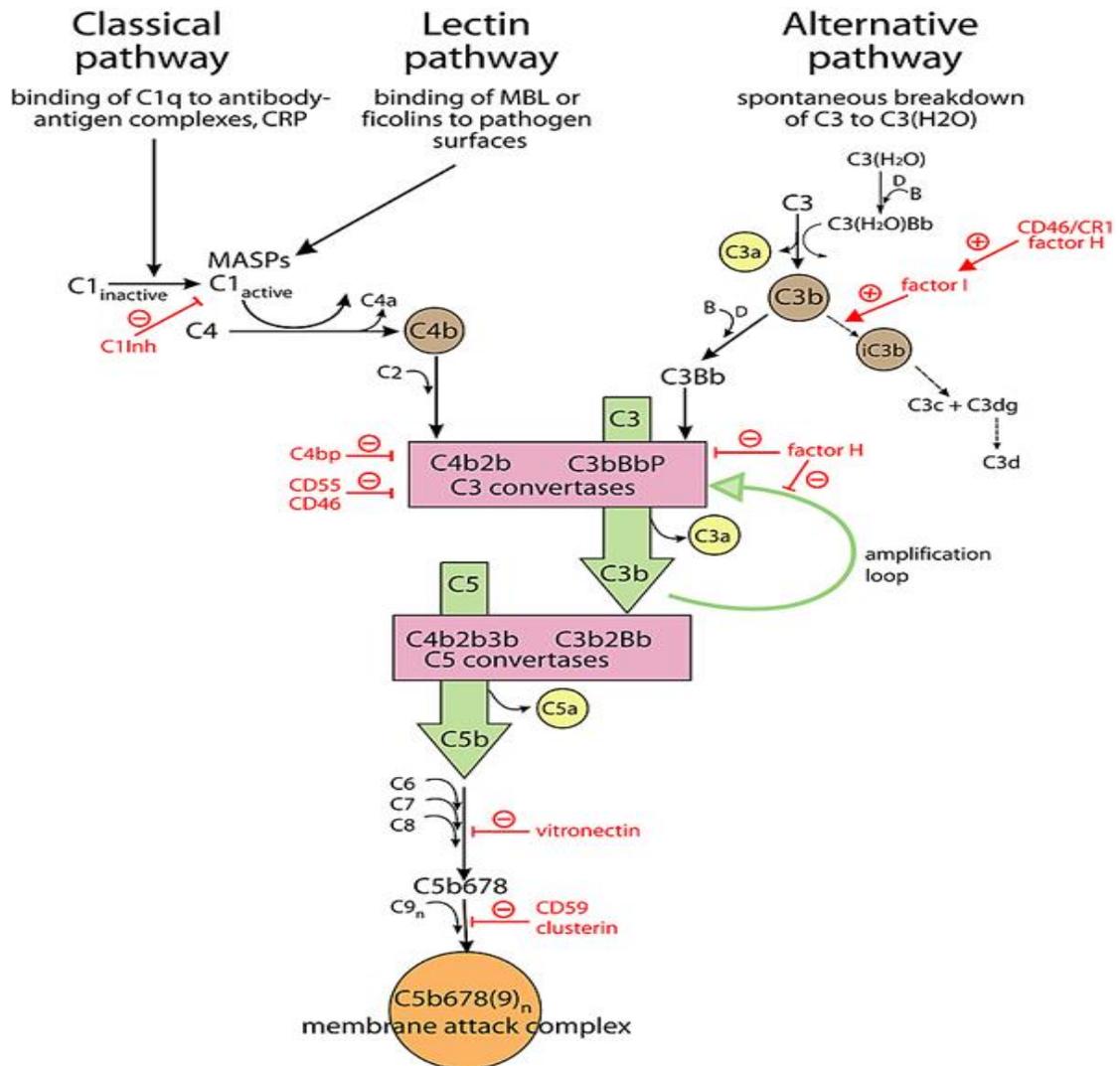
The highly conserved complement system of the innate immune response. The figure summarises the three major pathways through which complement is activated. Classical pathway, activated by binding of complement protein C1q to antibody:antigen complexes, the lectin pathway is activated by binding of serum mannose binding lectins to mannose expressed on the pathogen, and the alternative pathway does not depend on antigen binding complexes, but utilises the deposition of C3 complexes on target cells (Oksjoki et al., 2007)

1.1.1.2.3   Other granulocytes

Eosinophils are innate immune leukocytes characterised by specific granules found in their cytoplasm. They constitute 1-4% of circulating blood cells, are involved in

inflammatory responses and modulate the innate and adaptive immune system. They are triggered by activation of receptors for immunoglobulins, mainly IgE, cytokines and complement. Upon activation, they secrete an array of molecules including cationic granule proteins through degranulation. These proteins are cytotoxic to helminths, airway epithelium and have antimicrobial effects, regulate vascular permeability and modulate cellular trafficking across vascular membranes (Hogan S, 2008). Eosinophils are involved in the immune response to allergens, helminthic and parasitic infections. Activation of these cells is primarily by type 2 cytokines (released by a subset of T-helper cells, T_h2) IL-3, IL-5 and Granulocyte Macrophage- Colony Stimulating Factor (GM-CSF) (Yamaguchi et al., 1988, Asquith et al., 2008).

Although much work on eosinophils focuses on their role in parasitic infections and allergy, there are data to show that they are also effective against bacteria. Antimicrobial killing of bacteria is carried out by the release of superoxide in an NADPH dependent manner (Persson et al., 2001). Experiments by Linch *et al*, 2009 illustrated the *in vitro* anti-pseudomonal properties of mouse eosinophils. In transgenic mice secreting eosinophilic IL-5, improved clearance of *Pseudomonas aeruginosa* was observed (Linch, 2009). However in eosinophil-deficient mice, bacterial clearance was impaired; this improved upon transfer of eosinophils. Similar to neutrophils, eosinophils release extracellular trap efficient in trapping and killing bacteria (Yousefi, 2008).

Mast cells are haematopoietic cells that are dispersed throughout most tissues. Beyond their role in allergic responses, there is growing knowledge about the role and function of mast cells in the recognition of pathogens and modulation of immune responses. At the initiation of infection, mast cells are able to directly recognise PAMPs through expression of TLRs and Fc receptors (FcRs). Lipopolysaccharide

triggers TLR4 response in rodent mast cells for instance, and increased production of TNFα, IL-1β, IL-6, and IL-13 while peptidoglycan triggers TLR2 response with the induction of TNFα, IL-4 IL-5 and IL-13 responses (Fatehi et al., 2013). Apart from cytokine production, mast cells undergo degranulation in response to exogenous stimuli that accompany pathogen injection

1.1.1.2.5   Natural Killer (NK) cells.

NK cells are large granular lymphocytes phenotypically characterised by their expression of the CD56 adhesion molecule. They are rapid responders to a variety of infections, secreting chemokines and cytokines especially IFN-γ (Lanier, 2000). Although most research has focused on NK cells with regard to their antitumor and anti-viral activities, it is also evident that these cells are effective against bacteria and are activated preferentially by IL-12 (Kelly et al., 2002). NK cells can also be activated directly by bacterial products such as lipopolysaccharide (LPS) (Kirby et al., 2002) through recognition by TLRs, and by Gram-negative bacteria *Salmonella enterica* serovar Typhimurium, via specific glycolipids on the cell surface, including the glycosphingolipids; α-galacturonosylceramide and α-glucuronosylceramide (Brigl et al., 2003). This results in the induction of cytokines and co-stimulatory molecules, including IL-12 which stimulates IFN-γ production by NK cells.

Activation of NK cells can occur upon stimulation from cytokines such as IL-2, IL-12, IL-15, IL-18, IFNα or activating receptors such as natural killer group 2D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) (Chan et al., 2014). NKG2D activates NK cell mediated cytotoxicity following interaction with its ligands. In humans, the ligands for NKG2D are self-proteins related to MHC class 1 molecules and consist of the MHC class 1 chain-related protein (MIC) family and the UL-16 binding protein.

NKG2D ligands are generally expressed following cellular stress due to viral infection or malignant transformation and are absent on the surfaces of healthy cells. DNAM-1 is expressed on NK cells, T cells macrophages. Ligands for DNAM-1 are also expressed on virus infected and transformed cells. DNAM-1 has been shown to promote adhesion of NK cells and increase their cytotoxity against their targets (Chan et al., 2014).

1.1.2 Adaptive Immune Response

In order to survive in a host, pathogens devise various means of going undetected by the immune system. They have the ability to mutate their PAMPs in such a way that recognition is limited. Since the innate arm of the immune system is limited in the number of PAMPs it can recognise (Mogensen, 2009), an adaptive immune system is essential (Cooper, 2006). In contrast to the innate immune system where the recognition cascade has been encoded in a fully functional form within the germline genome, the adaptive system is tailored to generate flexible and specific responses by means of somatic recombination of gene segments. These specific and flexible responses to pathogens persist for life within the host, offering immunological memory and a more competent response in the case of a re-infection.

Effector mechanisms of the adaptive immune system include the T-cells and the B-cells, which undergo maturation in the thymus and bone marrow, respectively. Most often, adaptive immune responses originate under signals provided by the innate immune system directly or indirectly through circulating pathogens or pathogen activated antigen presenting cells (APCs), which capture antigen at the site of infection and migrate to the spleen and lymph nodes, where they present antigens to

lymphocytes. Adaptive immune responses develop in these sites and with the help of an array of chemokines and adhesion molecules, activated cells leave and traffic to the sites of infection (Sanchez-Madrid and Sessa, 2010).

1.1.2.1   T cells.

T cells play a central role in defining the functional and developmental responses of the adaptive immune system. They have a high specificity for antigens, generate immunological memory and can be recalled in the event of subsequent reinfection. According to their functions, T cells are classified into cytotoxic T cells (CTL), helper T cells (Th1, Th2, Th17, Tfh) and regulatory T-cells (Treg) (Hesslein et al., 2011). Based on their expression of either CD4 or CD8 co-receptors, cytotoxic T-cells are further classified as CD4^{+} T cells or CD8 [+] T cells. T cells recognise antigens in the form of peptides when displayed by major histocompatibility complexes (MHC) on the surface of antigen presenting cells (APCs). CD4 T cells recognise antigenic molecules displayed on the surface of MHC class II molecules, and the CD8 T cells recognise antigenic peptides from the cytosolic compartments displayed on MHC class I molecules. Activated CD8^{+} T cells induce the production of cytotoxic molecules including perforin and granzymes which enter target cells causing cell lysis. CD4^{+} T cells, on the other hand, in relation to antibacterial defence, influence the activities of B cells, and increase the antibacterial activities of phagocytic cells.

Th1 cells have been shown to play a central role in macrophage activation. These activated macrophages increase their antimicrobial activities among others, by production of superoxide and nitric oxide. Although these agents are effective antimicrobials, their activities are tightly regulated to avoid tissue destruction

(Duffield, 2003). T cells also have a role in responses to intracellular bacteria as well as in the induction of B cell responses.

1.1.2.2   B Cells.

B cells are effective mediators of humoral responses in the immune system and account for approximately 15% of circulating leukocytes (Chaplin, 2006). Development of these cells occurs through a carefully regulated process that differentiates hematopoietic precursor cells into transitional and immature B cells. They migrate to the lymph nodes and spleen where they undergo maturation into antibody producing plasma cells and memory B cells (Marcus et al., 2011).

B cells are uniquely defined by their production of antibody that acts as the B cell receptor (BCR). When BCR recognise foreign antigenic protein, fragment or molecule, they internalise, process and present these antigens to $CD4^+$ T-cells. This process promotes generation of plasma cells and development of a germinal centre. In this germinal centre, somatic recombination is up-regulated, leading to the development of a clonotypic B cell with increased affinity to the antigen. B cells at this point also undergo isotype switching. It has also been reported that engagement of bacterial LPS to B-cells tones up the capacity of these cells to produce the anti-inflammatory cytokine IL-10. This cytokine enhances the proliferation, survival of and antibody production in B-cells (Zhang et al., 2007).

Antibodies produced by B cells protect the host from pathogens in a number of ways; they neutralise the surface molecules of pathogens (toxins), preventing them from functioning effectively by binding to the toxins, neutralising their interaction with host cells. Antibody-antigen complexes are recognised and destroyed by macrophages. Since many bacteria have an outer membrane that is not recognised by the PRRs of

the innate immune system, they often evade the immune response such as TLR recognition. Antibodies coat these antigens in a process known as opsonisation, making them easily picked up and degraded by macrophages and neutrophils through FcR.

1.2   Antibiotics

The discovery and development of antibiotics is one of the major achievements of medicine and has greatly impacted on the quality of human lives. Antibiotics are an extremely important weapon in fighting infections. In animals and humans, the innate immune system produces its own antimicrobial peptides, which are evolutionarily conserved (Zasloff, 2002). Although by secreting antimicrobial peptides host defence mechanisms contribute to the elimination of certain bacteria, e.g. *Staphylococcus aureus*, *Escherichia coli* and *Mycobacterium leprae* (Izadpanah and Gallo, 2005), disease states arise where the host immune system becomes overwhelmed by the bacterial burden. Vaccines have been effective in reducing diseases that have affected humanity. However, the emergence of resistant strains of bacteria has increased the demand for development of more effective vaccines (Mortellaro and Ricciardi-Castagnoli, 2011). Though effective in generating protective immunity, treatment with vaccines has been associated with mild to severe side effects. Vaccines are generally very safe, but side effects from mild e.g. flu-like symptoms to severe e.g. Guillain-Barre syndrome following swine flu vaccination have been reported (Haber et al., 2009). Rare cases of reversion from attenuated strains to virulent forms lead to development of vaccine associated disease (Mills, 2009).

The ultimate aim of any antimicrobial therapy is to reduce morbidity and mortality associated with the infection (Wispelway, 2005) and antibiotics are usually administered to treat an infection. Antimicrobial agents are used to prevent infection

(when vaccines are not available), termed antibiotic prophylaxis. This prophylactic therapy has had substantial public health impact by reducing the disease burden associated with bacterial infection (Prasad and Karlupia, 2007). Antibiotic prophylaxis is also an optimal strategy to reduce wound infections in surgery, also known as surgical site infections (SSI) (Sikora and Koziol-Montewska, 2010). Surgical site infections are a common occurrence in colorectal surgery, due to the presence of a dense microbial flora in the colon. Antibiotic prophylaxis reduces the emergence of surgical site infections following colorectal surgeries (Ho et al., 2011). For effectual antibiotic prophylaxis outcomes, the choice of antibiotics to be used, surgical procedure to be carried out, time of administration of antibiotic and mode of administration are to be considered (Tammer et al., 2011). The eventual outcome of the infectious process and antibiotic therapy are both functions of multiple variables; the host defence response to the invading pathogen, intrinsic mechanisms within bacteria that help circumvent host defence, and the activities of the antimicrobial agent within the host. The interaction between the host and the invading microbe, elicited by the use of antimicrobial therapy is either advantageous or detrimental to the outcome of the infection (Yim et al., 2006).

1.2.1 Antibiotics and their therapeutic use.

1.2.1.1 Sulphonamides

These were the first class of antimicrobials, approved for the widespread treatment of infections in 1932 (Van de Velde et al., 2008). They are bacteriostatic and disrupt the synthesis of folic acid in bacteria. Sulphonamides are rapidly absorbed, metabolised and effectively excreted by the kidney (Reese and Betts, 1991). Sulphonamides are

derivatives of sulphanilamide, a compound similar to para-amino benzoic acid (PABA) found in bacteria. They target and inhibit the bacterial enzyme dihydropteroate synthetase (Figure 1.3) which catalyses the incorporation of PABA into dihydrofolic acid during folic acid synthesis. The sulphonamide is a competitive inhibitor and so reduces the amount of tetrahydrofolic acid, a co-factor in the synthesis of bacterial DNA (Patel et al., 2004). Sulphonamides are active against both Gram-positive and Gram-negative bacteria, including *Streptococcus pneumoniae, Corynebacterium diphtheriae* and *Haemophilus ducreyi* to mention a few*.* However, infections caused by *Proteus mirabilis, Enterobacter aerogenes and Pseudomonas aeruginosa* are no longer efficiently treated by sulphonamide antibiotics due to bacterial resistance to these agents (Eliopoulos and Huovinen, 2001).

Therapeutic use

Sulphonamide drugs are effective antimicrobial therapy for the treatment of uncomplicated, acute urinary tract infections. Single doses of a sulphamethoxazole-trimethromprim (a synthetic antibiotic and inhibitor of bacterial hydrofolate reductase) combination has been effective in treating women with cystitis (Nicolle, 2003).

Figure 1. 3 Sulphonamides are competitive inhibitors of dihydropteroate synthetase
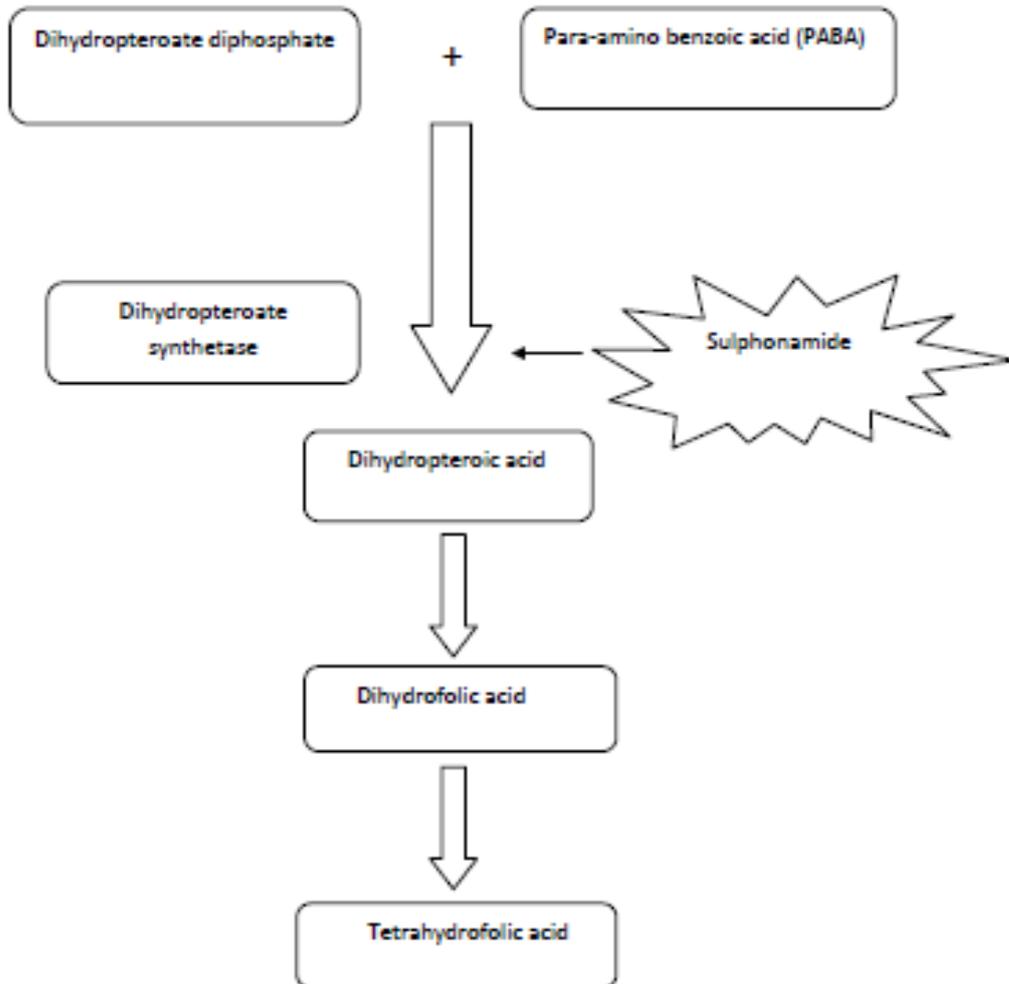
This combination is available commercially as co-trimoxazole, and is used in the prophylactic treatment of urinary tract infection and pneumonia (Garnero et al., 2010). Co-trimoxazole contains trimethoprim and sulphamethoxazole in a ratio of 1:5 (Aronson, 2006). The two compounds are less active alone, but in combination they work together in enhancing the activity of each other. Mechanism of action of trimethoprim involves inhibiting microbial reductases but its efficacy is improved in

the presence of a sulphonamide (Hitchings, 1973). The most common therapeutic use of co-trimoxazole is in the treatment of urinary tract infection (UTI) where it is administered as single-dose or conventional therapy i.e. 4 regular strength tablets (containing 80mg of trimethoprim and 400mg sulphamethoxazole) or two regular strength tablets twice daily, respectively. In severe cases of lower UTI and pylonephritis, co-trimoxazole is administered intravenously (Connor, 1998). Orally administered co-trimoxazole is effective in the treatment of respiratory tract infections, pneumonia and in cases of acute and chronic bronchitis, and is the drug of choice in the treatment of lung infections caused by opportunistic bacteria *Pneumocystis carinii*. The World Health Organisation and the Joint United Nations programme on HIV/AIDS in 2000 recommended co-trimoxazole prophylaxis for immunosuppressed patients and children born of HIV positive mothers (WHO, 2006, Sibanda et al., 2011). Co-trimoxazole is also recommended in prophylactic treatment of travellers' diarrhoea (Vila et al., 2001), a disease caused predominantly by *E. coli*, shigellosis and as a second line drug for salmonellosis.

In combination with erythromycin, sulphonamides are also used in treating acute and recurrent cases of otitis media caused by strains of *Haemophilus influenzae*, (Erramouspe and Heyneman, 2000), prophylactic treatment of meningococcal diseases (Connolly and Golden, 2011), as well as *Chlamydia trachomatis* infections, which is an intracellular pathogen. The WHO recommends co-trimoxazole for prevention of opportunistic bacterial infections and *Pneumocystis jirovecii* (WHO, 2014). The wide spread use of co-trimoxazole is correlated to resistance to this drug. Cornick and colleagues reported 92% resistance to co-trimoxazole in Malawians registered for co-trimoxazole preventive therapy (CPT) and have recommended the

re-evaluation of the prophylactic use of this drug against pneumococcal infections in HIV/AIDS patients in sub-Saharan Africa (Cornick et al., 2014).

1.2.1.2   Quinolones

The quinolone group of antibiotics have been used in clinical practice since the 1960s after the introduction of the first quinolone, nalidixic acid, in 1962. Traditionally, quinolones were used in treating infections arising from Gram-negative organisms, but the compounds have been modified and new agents are used for the treatment of specific infections with Gram-positive organisms (Oliphant and Green, 2002).

The quinolones act by inhibiting the synthesis of bacterial DNA. They cleave bacterial DNA in the DNA gyrase-type IV topoisomerase complex, resulting in the rapid death of bacteria (Hooper, 2000b). Quinolones kill bacteria in a concentration dependent manner. At about 30 times their minimum inhibitory concentration (MIC), the efficacy of these drugs is more pronounced, reducing bacterial activities by inhibiting the synthesis of RNA and proteins (Piddock, 2006). The quinolones unlike other antibiotic classes are not predictably synergistic when used in combination with other classes of antibiotics (Hooper, 2000b). In terms of absorption, administration of a quinolone orally is comparable to intravenously dosing (Walker, 1999). Quinolones are well absorbed with food, however, in the presence of cations such as magnesium, iron, calcium and aluminium, quinolones are chelated reducing their bioavailability, absorption, serum concentration and penetration (Turnidge, 1999). They have exceptional tissue penetration and also penetrate well into neutrophils and macrophages.

Quinolone antibiotics are classified into four groups according to their antimicrobial activity (Owens and Ambrose, 2000). The first group are the first-generation

quinolones, used less often as they have minimal systemic distribution and are only active against Gram negative bacteria. This group includes nalidixic acid, cinoxacin, and oxolinic acid (Skyrianou et al., 2011). The second-generation quinolones have extended activity for Gram negative bacteria, but are still limited in their activity for Gram positive bacteria. This second group is further sub-divided into class I (norfloxacin, enofloxacin, lomefloxacin and ciprofloxacin) and the class II (ciprofloxacin and ofloxacin). Class I differs from Class II in terms of tissue and intracellular tissue concentration (Oliphant and Green, 2002). Class II achieve higher drug concentrations in bacteria and are effective against atypical pathogens such as *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* (Blasi, 2004). Third generation quinolones (e.g. levofloxacin) retain Gram negative activity and have an improved spectrum of activity against Gram positive bacteria. The fourth-generation quinolones (e.g. moxifloxacin) are effective against Gram negative bacteria, have improved Gram positive activity and are active against anaerobic bacteria (Ambrose et al., 1997, Oliphant and Green, 2002, Owens and Ambrose, 2000).

Therapeutic use

Quinolones are effective in the treatment of uncomplicated urinary tract infections caused by *Escherichia coli*. Quinolones such as ciprofloxacin, lomefloxacin and levofloxacin have greater renal concentration and high renal clearance, hence, they are recommended for the treatment of complicated UTI such as catheter related infections, obstructive uropathies or stones due to *Candida species*, Gram positive and Gram negative pathogens (Hooper, 2000b, Johnson, 2002, Emonet et al., 2011). Since quinolones effectively penetrate prostatic tissue, they are recommended for the treatment of infection associated prostatitis, where the success rate in a four to six

week therapy is between 67-91% (Sabbaj et al., 1986). In first-line treatment of prostatitis, levofloxacin is recommended, however, in resistant Gram negative cases of prostatitis, enterococcal and pseudomonal prostatitis, ciprofloxacin is recommended as it has higher activity against enterococci and *P. aeruginosa* (Oliphant and Green, 2002). Quinolones have also been indicated for the treatment of acute bacterial sinusitis and community acquired pneumonia caused by *Streptococcus pneumoniae, Staphylococcus aureus* and *Haemophilus influenzae*. Pelvic inflammatory disease, a polymicrobial disease is also treated with quinolones. Ciprofloxacin is also considered in the treatment of *Neisseria gonorrhoeae* infections, enteric typhoid fever and travellers' diarrhoea (Hooper, 2000a). Though the quinolone class of antibiotics are highly successful, development of resistance has become a common outcome (Takahashi et al., 2003). Hence, it is recommended that quinolone antibiotics should not be used as first-line treatment for infections, as this strategy minimises the potential for development of resistance (Oliphant and Green, 2002).

1.2.1.3   Macrolides

Macrolide antibiotics are a group of antimicrobials whose activities are due to the presence of a macrocyclic lactone nucleus to which sugars are attached. The type and position of the sugar as well as the changes in the point of attachment determine the level of antimicrobial activity (Strausbaugh et al., 1976). Macrolides use dates back to the 1950s when erythromycin A was discovered as an alternative for the beta-lactams (Shinkai et al., 2008). Macrolides act by inhibiting the biosynthesis of bacterial proteins, via inhibition of ribosomal translocation, as well as preventing peptidyl transferase from adding the peptide in the tRNA to the next amino acid during the process of protein synthesis (Gaynor and Mankin, 2003). Macrolides also

reduce flagellin expression required for motility in *P. aeruginosa* (Kawamura-Sato et al., 2001).

Therapeutic use

Macrolides are used in the treatment of airway infective diseases. Diffuse panbronchiolitis (DPB) is a complex pulmonary disease affecting populations of Japan and Korea. It is characterised by chronic, progressively inflammatory and restrictive pulmonary function, sputum expectoration, dyspnea, and in severe cases bronchiectasis, (Bush and Rubin, 2003) and in most cases patients are infected with *P. aeruginosa* (Krishnan et al., 2002, Takeda et al., 1989, Kadota et al., 2003). Macrolides also exert unconventional effects on microorganisms such as *P. aeruginosa* where they inhibit twitching motility and inhibit biofilm formation (Bush and Rubin, 2003). Azithromycin is used in the treatment of *Mycoplasma genitalium* and single dosing is reported to be the best treatment of choice for this sexually transmitted infection (Jernberg et al., 2008). However, a recent publication has suggested that an extended regimen may be more effective in treatment of *M. genitalium* infections (Weinstein and Stiles, 2011). Clarithromycin and azithromycin are used for the treatment of *Helicobacter pylori*-associated peptic ulcer disease, sexually transmitted diseases and respiratory tract infections (Zuckerman et al., 2011).

1.2.1.4 Beta-lactams

Beta-lactam antibiotics are a broad class of antimicrobials, characterised by the presence of a beta-lactam ring in their chemical structure. They are the most widely used class of antimicrobials because they are effective against a wide range of bacteria, and have an excellent safety profile (Danziger and Neuhauser, 2011).

These antibiotics can be administered orally, parenterally or as inhaled dosage formulations. Beta-lactams include the penicillins, monobactams, cephalosporins, penems and carbapenems. They are active against anaerobic organisms, Gram-positive and Gram-negative bacteria (Mandell and Perti, 1996). Beta-lactams act by interfering with the cross linking of peptidoglycans which is needed for the structural formation of bacterial cell wall (Holten and Onusko, 2000). Due to their efficient absorption post oral administration, beta-lactams are very useful in the out-patient setting.

Therapeutic uses.

The penicillin group of beta-lactams still maintain their clinical relevance in the 21st century as haemolytic *Streptococci, Pneumococci* and oral anaerobes remain susceptible to penicillin. Benzylpenicillin is still the first choice antibiotic for the treatment of community acquired aspiration pneumonia, pneumococcal pneumonia, as well as streptococcal pharyngitis (Williams, 1993).

Cephalosporins were introduced for the treatment of staphylococcal infections but did not gain much favour against the penicillins. Incorporation of new side-chains into the chemical structure of these beta-lactams antibiotics improved their spectrum of antimicrobial activity against Gram-negative bacteria to include *Moraxella, Haemophilus, Pseudomonas* spp and Enterobacteriaceae (Williams, 1993).

Beta-lactams are more effective when they maintain a concentration above the minimum inhibitory concentration (MIC) of the invading pathogen. Once the antibiotics concentration drop to sub-MIC concentrations, the bacteria begin to proliferate rapidly (Vogelman et al., 1988). A common mechanism of resistance to beta-lactams is mediated by bacterial beta-lactamases. These enzymes attack the

beta-lactam ring, break it open, and thereby render the antibiotic properties of the drug inactive (Jovetic et al., 2010). Hence, beta-lactams are co-administered with clavulanic acid, a beta-lactamase inhibitor.

1.2.1.5 Linezolid

Linezolid belongs to the oxazolidinone class of antibiotics. Other members of this group include tidezolid, used for the treatment of acute skin infections, cycloserine, used in treating tuberculosis and posizolid under investigation for the treatment of *Staphylococcus aureus*, coagulase-negative staphylococci, Enterococcus spp., *Streptococcus pneumonia* (Wookey et al., 2004). Linezolid was developed at the Upjohn Company in the United States in a bid to meet the needs for newer antimicrobial agents. Linezolid has been used for the treatment of Gram-positive bacterial infections in the UK since 2000 (Gould, 2011). It works by inhibiting the synthesis of bacterial proteins. It is a highly effective antibiotic and competes favourably with vancomycin (Leach et al., 2011). Linezolid is indicated in the treatment of infections caused by *Streptococcus pneumonia,* vancomycin-resistant *Enterococcus faecium and* methicillin-resistant *Staphylococcus aureus (*Leach et al., 2011*).*

1.2.2 Efficacy of antibiotics *in vivo* vs. *in vitro*?

Routine antibacterial susceptibility testing methods, such as the determination of the minimum inhibitory concentration (MIC) of antibacterial dugs, have been used to test the activity of antibacterial agents on growing bacteria *in vitro* (Andrews, 2001, Matuschek et al., 2013, Hombach et al., 2011). Tissue culture and animal models have been used to understand and correlate *in vitro* activities of antibacterial drugs to *in vivo* activities. However, it is unclear whether anti-microbial activities observed *in*

vitro or in tissue culture accurately reflect activity *in vivo* (Brook, 1991). There are also discrepancies between the views of bacteriologists and immunologists in the context of how the terms 'in *vitro*' and '*in vivo*' are used. For the purpose of this chapter, '*in vitro*' refers to experiments not involving eukaryotic cells, '*in vivo*' refers to experiments carried out in animal/humans and '*ex vivo*' refers to experiments involving cells or samples obtained from animals or humans.

The effect of five antibacterial drugs (dicloxacillin, cefuroxime, gentamicin, azithromycin and rifampicin) on MSSA (E19977) was studied both *in vitro* (using time kill curves) and *in vivo* in a mouse peritonitis/sepsis model, using the maximum serum concentrations (C_{max}) of each drug. *In vitro* experiments showed that gentamicin incubated with 10^6 CFU/ml of *S. aureus* was most effective, achieving a four-log decrease in bacterial numbers after four hours of exposure. A slower, but more persistent effect was observed for rifampicin compared to cefuroxime and dicloxacillin. Azithromycin had the smallest bactericidal effect, but showed a bacteriostatic effect for the first eight hours. After twelve hours, bacterial re-growth occurred with all five antibacterial drugs tested. In the *in vivo* peritonitis mouse model, mice were challenged with bacteria (10^6 CFU/ml) and subsequently treated with antibacterial drugs two hours post bacterial challenge. Azithromycin showed poor antibacterial effects both *in vitro* and *in vivo.* Rifampicin, dicloxacillin, cefuroxime and azithromycin showed similar effects between their *in vitro* and *in vivo* studies, while gentamicin was most effective *in vitro* but less effective *in vivo* (Sandberg et al., 2009). Moreover, dicloxacillin was shown to be more effective against *S. aureus* in an *ex vivo* (THP-1 cell model) model than in an *in vivo* mouse peritonitis model. It was suggested that the reduced *in vivo* effect was due to processes such as the general elimination of the drug from the site of the infection and protein binding of the

antibacterial drug, and that the hostile *in vivo* environment could slow bacterial growth rate, leading to reduced anti-bacterial activity of dicloxacillin as it is only effective against actively dividing bacterial cells (Sandberg et al., 2010).

Considering that macrolides are known to accumulate to high levels within eukaryotic cells, a high intracellular effect may be expected (Carryn et al., 2003). Azithromycin has an accumulation level (the ratio of the intracellular concentration to the extracellular concentration (C_C/C_E)) of 40 to 300 at equilibrium compared to other macrolides with accumulation levels of between 4 to 50 (Van Bambeke et al., 2006). However, the same is not true for all antibacterial drugs. Although lower levels of accumulation within eukaryotic cells (e.g. macrophages) have been reported for beta-lactam drug such as penicillin, cefuroxime was effective against intracellular *S. aureus*, as the reduction in the viable count of bacteria was the same as *in vitro*. These findings suggests that the efficacy of an antibacterial drug *in vitro* is not always correlated to *in vivo* activity and may not always predict *in vivo* efficacy (Lin and Lu, 1997).

A number of factors are responsible for the increased efficacy of antibacterial drugs *in vitro* compared to *ex vivo* and *in vivo*. These include the drugs being located in different sub-cellular locations in eukaryotic cells such as macrophages or neutrophils and impaired intracellular activity of the antibacterial drug leading to bacterial resistance (Sandberg et al., 2009).

In vitro models are important because they help to translate research from the laboratory to clinical settings. In the *in vitro* model, bacterial number can be controlled, bacteria – antibacterial drug contact time regulated and the influence of environmental factors such as temperature, pH and oxygen standardised. However,

in vitro and *ex vivo* models do not closely mimic *in vivo* conditions as they do not account for host growth factors, proteases, antimicrobial peptides and fluctuations of the drug concentration due to tissue distribution and protein binding. Furthermore, bacteria behave differently *in vivo*, *ex vivo* and *in vitro*, hence this can account for the differences in the efficacy of antibacterial therapy under these different conditions (de Araujo et al., 2011).

1.2.3 Effects of antibiotics on immune cells

Antibiotic therapies target processes in bacteria, inhibiting their growth and eventually causing bacteria death. Ideally, antimicrobial agents should target invading microbes and have no effect on mammalian cells. However, it is inevitable that some antimicrobial agents that target processes including bacterial DNA and protein synthesis may affect similar processes in mammalian cells. Direct effects of antibiotics on immune responses and processes have been noted and studied for over 15 years (Tauber and Nau, 2008). Although described as "undesired effects" (Pasquale and Tan, 2005), reports have highlighted the therapeutic relevance of these antibiotic interactions with the immune system (Parnham, 2005).

Antibiotics can have immunomodulatory, immunosuppressive or neuroprotective effects (Lai and Todd, 2006). Pomorska-Mol and Pejsak described the immunomodulatory effects of antibiotics as "heterogeneous, contradictory or insufficient" as one antibiotic or class of antibiotic can have opposing effects when investigated using different methods or techniques (Pomorska-Mol and Pejsak, 2012). Of the studied effects, the immunomodulatory activities of antibiotics have been investigated most intensively, especially those of macrolides and quinolones. This is because these agents penetrate and accumulate in mammalian cells. Nonetheless, there is some evidence that other classes of antibiotics are potential

immune modulators (Gomez-Lus et al., 1997). Recently, the immunomodulatory ability of collistin in a nematode infection model was illustrated (Cai et al., 2014). It was reported that prophylactic administration of collistin protected *Caenorhabditis elegans* against infections from *Pseudomonas aeruginosa* and *Yersinia Pestis* by activating a conserved immune pathway, p38/PMK-1, in the intestines and transcription factors DAF-16 and SKN-1 required for resistance to pathogen infection (Cai et al., 2014). The effects of antibiotics on the immune response is discussed in the below, and a number of selected effects have been highlighted in Table 1.1.

1.2.3.1   Macrolides

Macrolide antibiotics are highly concentrated in the intracellular compartments of inflammatory and phagocytic cells. In the case of azithromycin for instance, accumulation of the antibiotic in innate immune cells enables delivery to the sites of infection (Parnham et al., 2014). Interest in the immunomodulatory activities of macrolides began in the 1960s, when their ability to complement corticosteroids in suppressing the immune response was observed after being administered to patients with severe asthma (Shinkai et al., 2008). In 2007, Piacentini et al., reported the ability of macrolides to reduce and regulate neutrophilic inflammation in patients whose asthma had been sustained by severe infections and patients with chronic steroid-resistant asthma. Short doses of azithromycin reduced airway infiltration of neutrophil, and bronchial hyper-responsiveness (Piacentini et al., 2007).

Macrolides decrease leukocytes adhesion to endothelial surfaces. Adhesion of leukocytes to endothelial surfaces is an essential step in the inflammatory process. This movement of leukocytes to sites of infections is mediated by chemotactic factors. Macrolides down-regulate this process by inhibiting the expression of

adhesion molecules on leukocytes. Sanz et al., carried out studies where lipopolysaccharide (LPS) infected rats were treated with erythromycin (30mg/kg) for a week. They showed that erythromycin significantly reduced the accumulation of elastase and neutrophils in the bronchiolar fluid produced in response to bacterial lipopolysaccharide (Sanz et al., 2004). Erythromycin also prevented the up-regulation of intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) in the vascular endothelium of mice in response to airway challenge with LPS. This inhibition of leukocyte infiltration was time dependent. Azithromycin decreased expression of adhesion molecules in untreated epithelial cells, but decreased expression of this molecule in epithelial cells or neutrophils stimulated with LPS. (Millrose et al., 2009, Parnham et al., 2014). Macrolides do not inhibit infiltration of blood leukocytes early in the inflammation process, rather cells that are primed by cytokines at the site of infection are restricted by this antibiotic and so ultimately contribute to the control of inflammation and prevent inflammatory damage to infected tissue (Culic et al., 2002).

Erythromycin affects interaction of bacteria with the host. Adherence of *P. aeruginosa* to Type 4 basement membrane collagen on acid-injured mouse tracheal epithelia was decreased when exposed to erythromycin (Tsang et al., 2003). Macrolides hindered the production of guanosine diphospho-D-mannose dehydrogenase (GMD) in the GMD cycle. This cycle allows for production of exopolysaccharide alginate and other virulence factors by *P. aeruginosa* (Wozniak and Keyser, 2004). Pre-incubation of human macrophages with erythromycin and roxithromycin decreased the phagocytosis of *Staphylococcus* (Carlone et al., 1989). Conversely, pre-treatment of *S. aureus* with macrolide drugs increased bacterial uptake by human macrophages. Beneficial anti-inflammatory activities of tilmocosin, a macrolide antibiotic used in the

treatment of bovine pneumonia, were reported with increased apoptosis of neutrophils, and phagocytosis by macrophages (Chin et al., 2000). Low doses of macrolides decreased bacterial LPS-induced production of IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF) by human bronchial epithelial cell line BET-1A (Desaki et al., 2004). In studies focusing on cellular immunity, macrolides also suppressed IFN-γ production by Th1 T cells and inhibits Th2 T cell functions in concanavalin A stimulated human T cells (Morikawa et al., 2002), as well as enhance regulatory T cell functions in a mice model of smoke induced lung inflammation (Bai et al., 2012, Altenburg et al., 2011). Further, azithromycin was also effective in modulating the increase in production of cytokines (interleukin [IL]-1beta (β), IL-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNFα) in chlamydial infections associated with infertility (Srivastava et al., 2009). Azithromycin has been shown to reduce pulmonary exacerbations in patients with cystic fibrosis after six months administration of the drug (Southern et al., 2012). The immunomodulatory effects exhibited by macrolide antibiotics especially azithromycin contribute to the resolution of acute infections and ameliorate the exacerbations in chronic airway diseases such as chronic obstructive pulmonary disease (COPD), cystic and non-cystic fibrosis (Parnham et al., 2014).

1.2.3.2   Fluoroquinolones

The first commercially available fluoroquinolone, norfloxacin was launched in 1980, and soon after that, its ability to modulate host immune responses was reported (Roche et al., 1987). Since these antibiotics accumulate within the host cell, they are able to attenuate virulence and pathogenicity of intracellular bacteria such as *S.* Typhimurium, *S. aureus* and *M. fortuitum* by preventing their proliferation (Dalhoff, 2005).

In human peripheral blood lymphocytes (PBL) stimulated with LPS, ciprofloxacin hindered the production of IL-1α and IL-1β (Bailly et al., 1990). These first generation fluoroquinolones influence the cytokine responses in leukocytes, but the type of fluoroquinolone, the concentration of the drug administered and the origin of the cells tested are important factors in determining the effect of fluoroquinolone antibiotics on leukocytes. Ciprofloxacin and rufloxacin are both inactive against *Bacteriodes fragilis in vitro*. By comparison, treatment of an intra-abdominal mouse model of infection with these antibiotics resulted in elimination of *B. fragilis* in 66.6% and 63.6% of animals, respectively. When TNFα levels were assayed, it was found that TNFα was lower in untreated mice than those administered with antibiotics (Gollapudi et al., 1993).

Chlamydia pneumoniae infections are marked by high inflammatory response, with increased levels of TNFα, MCP-1, IL-1, IL-8, and IL-6 (Vardhan et al., 2009, Kothe et al., 2000). Moxifloxacin is bactericidal against *C. pneumoniae* and modulates inflammatory responses via the NF-κB protein complex by inhibiting the degradation of IκBα, hence reduction in the production of inflammatory cytokines (Dalhoff, 2005). Moxifloxacin and sparfloxacin quinolones also increased IFN-γ levels in *Listeria monocytogenes* infections. IFN-γ is necessary for the activation of macrophages that phagocytose and clear these bacteria (Carryn et al., 2002). Furthermore, Webster et al., reported that in a granuloma model ciprofloxacin also caused a dose dependent inhibition of protein kinase C (PKC). This inhibition accounted for the activity of these drugs when administered to patients with inflammatory acne (Webster et al., 1994). Recently, Kalghatgi et al., showed that ciprofloxacin (as well as other bacteriostatic antibiotics such as ampicillin and kanamycin) increased intracellular reactive oxygen species (ROS) in human mammary epithelial cells by disruption of the electron

transport chain (ETC) (Kalghatgi et al., 2013). It was previously demonstrated in bacteria that exposure to bactericidal antibiotics led to production of lethal ROS by disruption of the tricarboxylic acid (TCA) cycle and ETC, which increases mutation rate hence giving rise to multi-drug resistant (MDR) bacteria (Kohanski et al., 2007, Kohanski et al., 2010). However, this ROS model has been challenged and disputed by various independent authors. For instance, Ricci et al., confirmed that ciprofloxacin exposure produced ROS in *S.* Typhimurium (L664 and SL1344) but did tender data to show that ROS production leads to selection of MDR strains (Ricci et al., 2012). Secondly it was shown that antibiotics were effective at killing bacteria but there was no evidence of oxidative stress (Liu and Imlay, 2013). Ezraty et al., suggested that cell membrane permeability other than oxidative was responsible for the selection of MDR and not ROS production (Ezraty et al., 2013). These studies show that ciprofloxacin exposure causes production of ROS in eukaryotic cells and in bacteria but does not promote the development of MDR bacteria.

In immunocompromised conditions such as HIV, patients are prone to infections such as salmonellosis. Both *S.* Typhimurium and HIV are intracellular pathogens and can replicate within monocytes and macrophages, although HIV replicate mainly in T cells. *S.* Typhimurium enhances the replication of HIV by inducing the production of TNFα. TNFα activates HIV-1 replication on T cells via translocation of NF-κβ to the nucleus and activation of HIV DNA long terminal repeat. Following ciprofloxacin treatment, intracellularly available ciprofloxacin causes bacterial death, reducing the numbers of *S.* Typhimurium within the cell. This effect leads to the inhibition of TNFα synthesis and subsequently hinders the replication of HIV virus within the macrophages (Andreana et al., 1994).

1.2.2.3 Tetracyclines

Tetracyclines display several activities on immune cells. Collagenase activities are induced during an immune response by cytokines and unregulated activity of these enzymes indirectly leads to tissue damage (Suomalainen et al., 1992). Tetracyclines also inhibit the zinc-dependent activity of metalloproteinase enzymes by chelating zinc from their active sites. Doxycycline in an *in vitro* experiment, inhibited leukocyte function and adhesion via divalent cation binding (Tauber and Nau, 2008). Tetracyclines enhanced survival of mice undergoing LPS-induced septic shock, by inhibiting inflammatory cytokines such as TNFα and interleukin-1 alpha (IL-α) produced by peritoneal macrophages. Tetracyclines inhibit the p38 mitogen-activated protein kinase (MAPK) and NF-κB pathways which are important in controlling the expression of proinflammatory mediators. Apart from immunomodulation, tetracycline is also involved in immunosuppression as well as neuroprotection. Minocycline delays the onset of neurodegenerative disease and slows its progression by preventing microglial activation, reducing induction of caspase 1 and decreasing levels of IL-1β a pro-inflammatory cytokine (Kriz et al., 2002).

In a C57BL/10 mouse model of *Chlamydia* infection, doxycycline intervention was very effective in rapidly reducing shedding and in eradication of the infection. However, on assessing the effect of doxycycline on antibody responses in the sera and vaginal washes of antibiotic treated mice, more IgG responses were found in the washes of untreated mice while anti-chlamydial IgA antibodies were undetectable in treated mice. There was also a reduction in IFN-γ and IL-10 production by CD4$^+$ Th1 cells in treated mice than untreated mice. This suggests that the although doxycycline negatively affected development of antibody and cell mediated immunity, this antibiotic maintained its efficacy against *Chlamydia* (Su et al., 1999).

1.2.3.4 Beta-Lactams

Relatively little data exist about the immunomodulatory activities of beta-lactams and these agents seem to lack anti-inflammatory activities (Tauber and Nau, 2008). Cefaclor is probably the most widely studied antibiotic in this group. It has been shown to enhance phagocytosis and potentiate bactericidal activities by shifting the immune response towards the Th1 responses (Periti, 1998). To study the effect of beta-lactams on IFN-γ activity, the lung epithelial cell line A459, was incubated with a range of concentrations of benzylpenicillin, D-penicillinamine, ampicillin, phenoxymethylpenicillin, cefotaxime, cefaloridine, cefoxitin, ceftriaxone, aztreonam, merepenem and clavulanic acid for 1 or 4 days. IFN-γ activity was measured by its ability to induce the expression of the cell surface phosphatase CD54 (ICAM-1) on human epithelial cells. Results showed that clavulanic acid, cefoxitin and cefaloridine had the greatest inhibitory effect on IFN-γ activity while penicillin and aztreonam had the least effect. The inhibitory effects of these antibiotics were time dependent, such that when IFN-γ was co-incubated with antibiotics from 1 to 4 days, the influence of the drugs became more pronounced (Brooks et al., 2005).

Mor and Cohen (2012) reported that experimental autoimmune encephalomyelitis and adjuvant arthritis were more severe in mice treated with oral cefuroxime. Transcriptome analysis showed genes involved in Th2 and T regulatory cell differentiation were reduced by cefuroxime, but up regulated in the presence of ampicillin (Mor and Cohen, 2012). Antibacterial drugs can not only affect the immune cells directly, but change to the gut microbiome alters the outcome of autoimmune diseases. Administration of broad spectrum antibacterial drugs (ampicillin, vancomycin, neomycin and metronidazole), that reduce gut microflora of C57BL/6 mice impaired the development of experimental autoimmune encephalomyelitis

(EAE) (Ochoa-Reparaz et al., 2009). Reduction in severity of EAE was associated with a reduced production of pro-inflammatory cytokines and increased production of IL-10 and IL-13 and increased production of certain B cell subpopulation e.g. CD5^{+} B cells. Transfer of these B cells to non-antibacterial treated EAE mice induced a shift from Th1/Th17 type responses towards anti-inflammatory Th2 type responses such as enhanced production of IL-10 and IL-13, and reduced production of IL-17 and IFN-γ (Ochoa-Reparaz et al., 2009, Ochoa-Reparaz et al., 2010). The difference in the observations in these studies was attributed to the binding of beta-lactam antibiotics including cefuroxime and penicillin to albumin. Transcriptome analysis showed that the expression of several genes was altered, in particular in the TGF-β pathway, which is involved in the signalling of both pro-inflammatory Th17 and anti-inflammatory Tregs. The half-life of penicillin bound to albumin was 7 days compared to 42 minutes for free penicillin, therefore Mor and Cohen postulated that any effects of bound albumin would be sustained (Mor and Cohen, 2012). However, why certain beta-lactams induce inflammation and others such as ampicillin are protective is not yet understood.

Finally, exposure of mice challenged with EL4 lymphoma, MC38 colon carcinoma and B16 melanoma cells to antibacterial drugs that clear the gut microbiota induced a down regulation of genes related to inflammation, antigen presentation, phagocytosis and adaptive immune responses. Genes related to cancer, tissue development and metabolism were upregulated in mice treated with antibacterial drugs. Antibacterial treatment led to a poor response to anti-tumour therapy than in untreated mice (Iida et al., 2013). These findings suggest that the normal gut microbiota is essential for optimal immune responses, and modulation of the microflora by exposure to antibacterial drugs may be detrimental to the outcome of some cancer treatments.

Antibacterial drugs also affect other mammalian cells by altering cellular functions such as mitochondrial production of reactive oxygen species (ROS). In a recent study, clinically relevant concentrations of four antibacterial drugs of different classes (ciprofloxacin, tetracycline, ampicillin and kanamycin) induced dose dependent increases in ROS production in a human mammary epithelial cell line (MCF10), human mammary epithelial cells (HMEC), human gut epithelial cells (CACO-2), and porcine aortic endothelial cells (PAEC) in tissue culture (Kalghatgi et al., 2013).

Table 1.1 Selected effect of antibiotics on the immune response.

Antibiotic class	Effect on immune cells
Macrolide	Inhibited neutrophilic inflammation in patients with severe infection and steroid resistant asthma (Piacentini et al., 2007), enhanced neutrophil killing (Labro et al., 1993)
	Improved clinical symptoms of acute sinusitis by decreasing nasal fluid IL-8, and serum levels of IL-1, 8 and c-reactive proteins (Labro, 1998).
	Inhibited the expression of adhesion molecules intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) on leukocytes (Sanz et al., 2004).
	Reduced phagocytosis in erythromycin treated mice (Carlone et al., 1989)
	Reduced number of neutrophils and inflammatory cells (Yamada et al., 2013)
	Ameliorated LPS induced inflammation in a rat model of acute conjunctivitis (Fernandez-Robredo et al., 2013).
	Reduction in LPS induced inflammatory cytokines and plasma kynurenine (Hao et al., 2013)
	Modulate the production of (interleukin [IL]-1beta (β), IL-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNFα)in response to Chlamydia
Fluoroquinolones	Inhibited IL-1α and IL-1 β(Bailly et al., 1990), induced overexpression of efflux transporters in J774 macrophages (Caceres et al., 2013)
	TNFα, MCP-1, IL-8, and IL-6 in Chlamydia infection
	Reduced inflammatory cytokine in human bronchial epithelial cells (Tsivkovskii et al., 2011)
	Inhibited intestinal inflammation in an IL-10 dependant manner (Gustot, 2014)
	Induced increases in intracellular reactive oxygen species (ROS) in human mammary epithelial cells (Vardhan et al., 2009, Kothe et al., 2000, Kalghatgi et al., 2013)
Tetracyclines	Inhibited both collagenases and metalloproteinase (Suomalainen et al., 1992, Kriz et al., 2002, Tauber and Nau, 2008, Su et al., 1999).
	Inhibited leukocyte function and adhesion
	Hindered development of antibody and cell mediated immunity
Beta-lactams	Clavulanic acid, cefoxitin and cefaloxidine inhibit IFN-γ activity (Brooks et al., 2005)
	Increased oxidative burst in neutrophils in response to P. aeruginosa (Labro et al., 1988)
	Facilitated the development of EAE and increased arthritis scores in mice (Mor and Cohen, 2012)

1.3 Antibiotics: better in the immunocompetent host or less so in the immunocompromised?

An immunocompromised patient is one who is unable to resist infection in a normal manner as a result of impaired host defences (Rolston, 2004). Infections are one of the major causes of death in immunocompromised individuals (Greenberg, 2002, Neumann et al., 2013). Defects in any aspect of the host defence leads to increased susceptibility to invading pathogens (Algar and Novelli, 2007). A compromised immune system can be due to congenital deficiencies (in neutrophils e.g. chronic granulomatous disease, antibody e.g. glycerol kinase deficiency, complement or T-cell deficiencies e.g. DiGeorge Syndrome), or acquired including administration of immunosuppressive drugs in patients undergoing organ/tissue transplantation, anti-TNFα therapy, chemotherapy or radiotherapy in cancer patients and disease states such as HIV infection.

The types of infections that are predominant in immunocompromised patients are usually reflective of the underlying immunodeficiency as defence against a particular microbe or microbial species is often dependent on a particular arm of the immune response. For instance, infections in neutropenic patients are commonly caused by *S. aureus*, *P. aeruginosa* and *S. pyogenes*. HIV patients are usually predisposed to infection caused by *Salmonella* species, *S. pneumoniae* and *Mycobacterium* species. (Shenep, 1998, Algar and Novelli, 2007). Bacterial pathogens which commonly cause infections in immunocompromised conditions are listed in Table 1.2.

Notwithstanding the small number of patients, it can be inferred from studies on patients with granulocyte deficiencies that the state of the immune system affects the outcome of antibacterial therapy. Administration of antibacterial prophylaxis reduced infection and early death in these patients as compared to administration of

antibacterial therapy once an infection was established (Drayson, 2011). Studies on linezolid (an oxazolidone antibacterial drug) in clinical trials investigated the efficacy and safety for the treatment of infections in cancer patients with neutropenia.

Table 1.2 Common infective pathogens associated with immune defects

Immunodeficiency	Bacterial species that commonly cause infections.	References
Neutropenia (due to chemotherapy)	*Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Pseudomonas aeruginosa, Klebsiella species.*	(Donowitz et al., 2001, Rolston, 2004)
Organ/tissue transplantation	*Listeria monocytogenes, Mycobacterium tuberculosis, Nocadia asteroides, Salmonella* species*, Legionella* specie, *Campylobacter jejuni.*	(Fishman and Rubin, 1998, Singh and Paterson, 1998) (Ampel and Wing, 1990, Dhar et al., 1991, Holden et al., 1980, Wilson et al., 1989)
HIV	*Salmonella* species*, Mycobacterium* species*, Streptococcus pneumoniae, pneumocystis carinii, Cryptosporidium* species*.*	(Crump et al., 2011, Lawn et al., 2005, Dayan et al., 1998)
Complement	*S. pneumoniae, Haemophilus influenzae, Neisseria meningitides.*	(Ram et al., 2010)

In immunocompromised adults with cancer, the efficacy of linezolid was 79%-86% (Smith et al., 2003). In immunocompromised children (average age, 2.2 years) with cancer (lymphoblastic leukaemia, brain tumour, multi-organ Langerhans cell histiocytosis, rhabdomyosarcoma, Burkitt's lymphoma and ovarian tumour), it was 100% (Moschovi et al., 2010). However, the efficacy of linezolid in children with bacterial infection was similar to immunocompromised children with cancer at 90% (Wang and Hsueh, 2009). These data suggest that linezolid was more effective in immunocompromised children with cancer than in immunocompromised adults. Notwithstanding the possibility that a greater number of infections in immunocompromised children were by pathogens susceptible to linezolid, contrary to the long-held assumption that a competent immune system is required for antibacterial drugs to be effective, these findings suggest that antibacterial drugs are capable of resolving an infection with or without a competent immune system.

1.4   Immunomodulatory antibiotics for treatment of bacterial infections; the way forward?

With the rate of discovery of new antibacterial drugs declining and the global increase in resistant bacteria (Piddock, 2012), the ability of antibacterial drugs to modulate immune responses either by suppression or enhancement may be a useful therapeutic strategy to be exploited for the treatment of infectious diseases (Hancock et al., 2012, Hawn et al., 2013). Focus needs to be drawn to promote the use of therapies that minimise the selection of resistant bacteria and agents that can be used alone or in a combination to moderate elements of the host response to reduce bacterial survival (Spellberg et al., 2013).

Several pre-clinical research programmes have demonstrated that by blocking the host's microbial receptors or signalling pathways, control of bacterial infection can be

achieved. One study for instance, demonstrated how the combined use of antibacterial drugs, and calcium and potassium ion flux inhibitors or a phenothiazine (an anti-psychotic drug) to target *Mycobacterium tuberculosis* infected macrophages enhanced the ability to kill internalised bacteria (Martins et al., 2008). They suggested that targeting the host's immune response could reduce the prevalence of multi-drug resistance tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR-TB) (Martins, 2011). Additionally, it was shown that α-galactosylceramide activated invariant natural killer T cells reduced replication of *M. tuberculosis* in mouse peritoneal macrophages and prolonged survival of infected mice. Apart from modulation of the immune response, it was shown that a combination therapy of α-galactosylceramide and isoniazid had a synergistic effect in controlling bacterial growth (Sada-Ovalle et al., 2010).

Toll-like receptors (TLR) recognise pathogen associated molecules and several immunomodulatory therapies that target TLR signalling are currently under investigation. A recent study in mice showed that administration of a broad spectrum antibacterial drug combination (metronidazole, neomycin and vancomycin) led to increased colonisation of the gut when challenged with vancomycin-resistant *Enterococcus* (VRE) due to reduced production of RegIIIγ, a secreted antibacterial C-type lectin which acts selectively on Gram-positive bacteria. Following administration of TLR4 ligand, lipopolysaccharide, RegIIIγ expression was restored in treated mice and the number of viable VRE in the gut was reduced. Similarly, administration of the TLR5 ligand flagellin to mice also reduced the viability of VRE in the gut, and may even be a better therapeutic option to LPS, as it does not induce a severe inflammatory response. These data suggest that mucosal resistance to bacteria can

be enhanced by agents that boost mucosal immunity. (Brandl et al., 2008, Kinnebrew et al., 2010).

The use of combined antibacterial therapy with enhancers of the innate immune system has been proposed (Sparo and Sánchez Bruni, 2012). One study demonstrated the improved therapeutic effect (80%) of *Mycobacterium phlei* cell wall extract (MCWE) which targets the TLR pathway, in combination with the fluoroquinolone enrofloxacin, in *Streptococcus zooepidemicus* infected mares who have previously failed treatment with MCWE alone (Fumuso et al., 2004). Such an approach involved recognising components of the immune response e.g. phagocytes as drug delivery vessels to sites of infection.

Jareoncharsri et al., (2003) showed that administration of two oral doses of Luviac®, a bacterial lysate agonist for TLR2 which contains antigens of seven bacteria common in respiratory tract infections (*S. aureus, Streptococcus mitis, S. pyogenes, S. pneumoniae, Klebsiella pneumoniae, Moraxella catarrhalis, Haemophilus influenzae*) over a four month period to patients with recurrent respiratory tract infection effectively reduced severity and duration of infection. Increased production of T lymphocytes (CD4, CD8 and CD45RO$^+$ memory cells), higher levels of specific IgA, and a rise in serum levels of IL-1β and IL-6 was noted in healthy volunteers treated with Luviac® (Jareoncharsri et al., 2003).

By comparison, Toll like receptors can also be modulated using antagonists that block the signalling cascade. OPN-305, a TLR2 antagonist developed by Opsona Therapeutics has been shown to block TLR2, thereby reducing TLR2 mediated pro-inflammatory cytokine production during myocardial ischemia/reperfusion injury (Connolly and O'Neill, 2012). OPN-305 was successful in Phase I clinical trials and

the company is currently recruiting participants for Phase II trials (Reilly et al., 2013). Phase II clinical trials of eritoran (E5564), an antagonist that inhibits endotoxin interaction with TLR4 developed for the treatment of sepsis (severe inflammatory response to infection, commonly bacterial infections), revealed that eritoran was well tolerated in volunteers (Tidswell et al., 2010). Phase III clinical trials however revealed that eritoran did not perform better than pre-existing anti-sepsis therapy and clinical trials were halted (Opal et al., 2013). A more recent study illustrated that eritoran prevented influenza lethality in wild type C57BL/6J mice, suggesting a novel approach in the treatment of influenza infection (Shirey et al., 2013). The used of TLR targeted therapeutics, both agonists and antagonists, for the treatment of infections, cancers and allergic disease has been extensively reviewed (Connolly and O'Neill, 2012).

Innate immune cells such as leukocytes and epithelial cells produce immunomodulatory and anti-infective compounds known as innate defence regulator (IDR) peptides (Hancock et al., 2012). One such peptide is hLF1-11 derived from the N-terminus of human lactoferrin, with potential clinical application in the prevention of infections caused by methicillin-resistant *S. aureus* (MRSA), *Listeria monocytogenes* and antibacterial resistant *Acinetobacter baumannii* in immunocompromised patients (Velden et al., 2009). Exposure of human monocytes to hLF1-11, during GM-CSF-driven differentiation, increased responsiveness to microbial structures such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and enhanced efficacy against MRSA and *C. albicans*. hLf1-11 was shown to bind to, and penetrate human macrophages, and subsequently inhibited the production of myeloperoxidase in the macrophages (van der Does et al., 2010, van der Does et al., 2012). However, there is no evidence that this drug advanced to clinical trial

(http://clinicaltrials.gov/show/NCT00430469). In addition to designing therapeutics that target specific pathways/molecules in bacteria, antibacterial drug adjuvants can be designed to target components of the host-pathogen relationship (Nathan, 2012). Since pathogens often require host factors for their persistence, an antibacterial drug that has the potential to inhibit the expression and action of such factors (e.g. kinases and phosphatases) might constitute an adjunct anti-infective therapy.

It has been reported that the absence of dsRNA-dependent protein kinase R (PKR) benefits the intact mammalian host during infection with *M. tuberculosis.* Mice deficient in PKR had a sustained reduction in the number of infecting bacteria and lung pathology compared to wild type mice. This protective effect of PKR deficiency was accredited to two mechanisms: (1) PKR deficiency enhanced apoptosis in macrophages infected with *M. tuberculosis*; and (2) PKR induces the expression of IL-10, a macrophage deactivating cytokine. In a PKR deficient environment, macrophage activation in response to interferon-γ (IFN-γ) enhanced the production of protective reactive nitrogen intermediates (RNI) compared to wild type mice (Napier et al., 2011, Wu et al., 2012). Treatment of mice with a protein kinase inhibitor, imatinib, pre- and post-infection reduced the growth of *M. tuberculosis* over one month. In addition to the functions stated above, PKR is an important signalling molecule involved in diverse physiological processes such as response to stress, cytokines, lack of nutrients and osteoblast mediated calcification (Sadler and Williams, 2007). Therefore, it is important to consider these other processes if administration of a drug that enhances the inhibition of PKR is proposed in a clinical setting.

Manipulation of intracellular signalling molecules derived from fatty acids may also be beneficial to an infected host (Nathan, 2012). This was illustrated in a C57BL/6J

mouse peritoneal model of *E. coli* infection, where there was increased survival of the mice following treatment with ciprofloxacin supplemented with anti-inflammatory metabolites of ω-3-eicospentaenoic acid or docosahexaenoic acid (resolvins and protectins) (Chiang et al., 2012). In addition to intracellular signalling fatty acid molecules, pro-resolvin lipid mediators have been shown to have immunomodulatory activities. Molecules such as resolvins, lipoxin, protectins and maresins are agonists of resolution, with the potential to limit infiltration of neutrophils and enhance the uptake of apoptic cells by macrophages and microbial clearance (Serhan, 2014). Together these studies illustrate that targeting host responses lowers the requirement for, and dependence on, antibacterial drugs that directly target bacteria, hence providing an alternative approach to address antibacterial resistance. Understanding the complex relationship between the effect of antibiotics on bacteria and immune cells provides an opportunity to develop novel therapies. By combining these effects we can potentially tailor the immune response to pathogens while inhibiting growth of the bacteria. This would lead to reduced antibiotic use and therefore less opportunity for resistance to develop, giving such drugs a longer therapeutic life.

1.5   Models of the immune response to infection.

Handel et al., (2008) proposed four mathematical models for the design of experimental tests. Handel and colleagues based their analysis on *in vitro* studies and mouse models of infection (Campion 2005 and Chung 2006). These studies modelled neutrophil and CD8+ T cell responses to bacterial infection. With regards to humans, the basic concepts outlined should remain the same, though, differences in metabolism may alter the specific parameters of the models (Craig, 1998). However, while preclinical data from murine experiments have been used to define treatment of

human patients, the assessment of risk should be more fully addressed (Lamontagne et al., 2010). The models were based on the following assumptions: (1) that immune responses are triggered at the onset of an infection and these responses saturate when they attain maximum strength to tackle the infection, (2) that immune responses against bacteria saturate as the bacterial load increases, as observed experimentally in neutrophils (3) that immune responses to an infection are proportional to bacterial growth and these responses deteriorate at a constant rate, for instance in cytokine responses and (4) a combination of assumptions (2) and (3) (Figure 1.4) (Handel et al., 2008). This was exemplified in animal studies such as the non-neutropenic mouse thigh models of *Pseudomonas aeruginosa* and methicillin-sensitive *Staphylococcus aureus* (MSSA) infections. Drusano et al., (2010) showed that granulocytes kill bacteria up to a given saturability, above this the bacterial load cannot be controlled. For *P. aeruginosa*, a bacterial challenge of 1×10^6 CFU/ml resulted in stasis (i.e. net granulocyte kill with no bacterial growth or death) whereas when challenged with 3×10^6 CFU/ml there was uncontrolled growth after 24 hours. For *S. aureus* infection a bacterial challenge of 3×10^6 CFU/ml resulted in bacteriostasis; when challenged with 1×10^7 CFU/ml there was bacterial growth (Drusano et al., 2010). The existence of a granulocyte killing saturability, therefore, increased the need for antibacterial chemotherapy to control bacterial growth, since unchecked bacterial growth caused severe damage morbidity and mortality. However, although these models are helpful in designing experimental strategies, they might not be directly applicable to animal models or people with impaired immune function which may also contribute to the development of resistance to antibacterial drugs during an infection (Handel et al., 2008, Borody et al., 2002).

Animal and *in vitro* models have been used to simulate *in vivo* conditions when studying immunity and how it affects antibacterial therapy. Bakker-Woudenberg et al., 1979 demonstrated the importance of the host's immune response during antibacterial therapy by administering penicillin to rodents with a selectively impaired immune system. Their experiments were carried out in a rat model of impaired phagocytosis induced by complement depletion, challenged with a *Streptococcus pneumoniae* type III strain and intramuscular administration of penicillin. Their results showed faster recovery from pneumococcal infection with penicillin therapy in rats with an intact complement system (100%) compared to complement-depleted rats (43%) (Bakker-Woudenberg et al., 1979). Dalhoff (2005) investigated the activity of two fluoroquinolone drugs, ciprofloxacin and moxifloxacin, against a clinical isolate of *P. aeruginosa* (308039) and a laboratory generated strain of *P. aeruginosa* (19397) in a pharmacodynamic mouse model devoid of host defence factors such as polymorphonuclear neutrophils (PMNs). The results showed that introduction of immunocompetence into the system by addition of J774 macrophages or by an *ex vivo* sponge soaked in inflammatory exudates caused an increase in bacterial killing. However, it was difficult to determine whether J774 macrophages increased the bactericidal activity of the drug or the J774 cells prevented re-growth of bacteria (Dalhoff, 2005).

The penetration, intracellular accumulation and distribution properties of antibacterial drugs such as macrolides and fluoroquinolones, improved their activities especially when infections by intracellular pathogens such as *S. aureus* were examined (Tulkens, 1991). Although it was disputed that *S. aureus* is an intracellular pathogen, it has been shown that *S. aureus* invades non-phagocytic cells such as epithelial cells, endothelial cells and fibroblasts (Garzoni and Kelley, 2009, Fraunholz and

Sinha, 2012). The effect of antibacterial therapy on macrophage engulfing *S .aureus* was explored in a J774 macrophage tissue culture model exposed to fluoroquinolone drugs (ciprofloxacin, levofloxacin and moxifloxacin) and subsequently infected with *S. aureus.* The results showed that macrophages exposed to these drugs had fewer engulfed bacteria than untreated (Seral et al., 2005). McDonald and Pruul suggested that macrolide drugs such as azithromycin work in synergy with the immune system and have potent immunomodulatory activities (McDonald and Pruul, 1992). Similarly, dirithromycin, another macrolide drug, reportedly enhanced phagocytic activity of neutrophils (Labro et al., 1993). Conversely, three macrolides (erythromycin, azithromycin and clarithromycin) were shown to inhibit the ability of neutrophils to generate reactive oxygen species. It was hypothesized that the clinical relevance of this inhibition would be the control of inflammatory processes in chronic respiratory diseases, especially if high concentrations of the drug accumulate in tissues (Wenisch et al., 1996, Sugihara, 1997).

Figure 1.4 Models of the immune response as proposed by Handel et al (2008).

A. Model
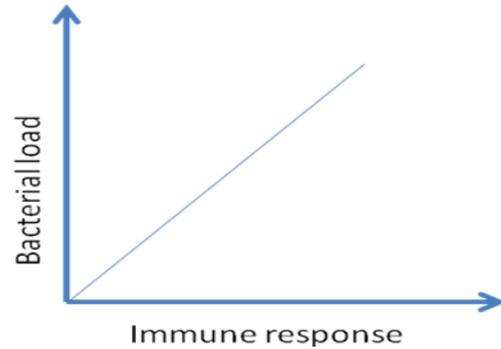
B. Model
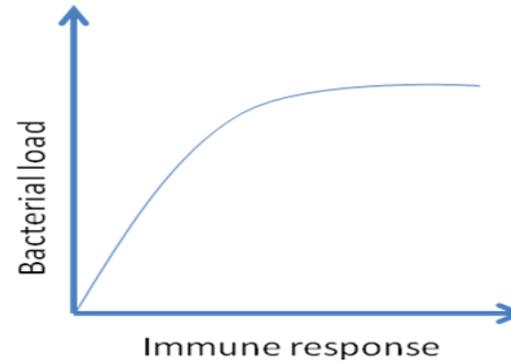
C. Model
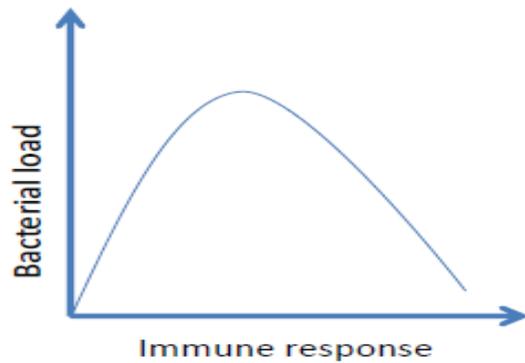
D. Model
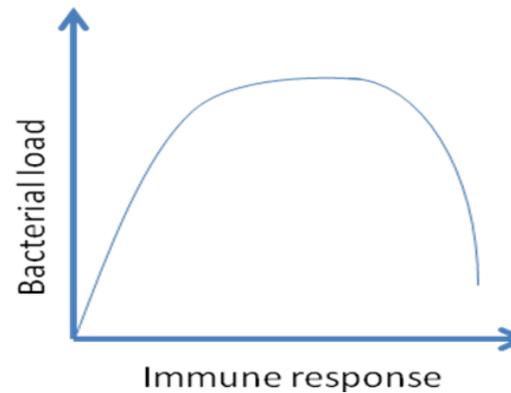
A. Model 1: The immune response is triggered at the onset of an infection. Bacterial killing is directly proportional to the strength of the immune response. This applies to neutrophils and CD8 cells and bacteria **B.** Model 2: The rate of bacterial killing saturates at a maximum level as bacterial load increases. This has been observed experimentally in neutrophil response to bacteria. **C.** Model 3: The immune response increases with bacterial load at a fixed rate e.g. in the cytokine response to bacterial infection. **D.** This model combines the saturated killing of model 2 and the dynamic of model 3.

1.6   Immune responses to *Salmonella* infection

Salmonella enterica serovar Typhimurium belongs to a class of enteropathogenic bacteria, responsible for a number of diseases. These diseases range from local gastroenteritis, to systemic life-threatening disease such as, typhoid fever (Fierer and Guiney, 2001).   *Salmonella* is a facultative anaerobic, rod-shaped Gram-negative bacteria belonging to the family of Enterobacteriaceae. Within the *Salmonella* genus there are two known species; *Salmonella enterica* and *Salmonella bongori* (Dougan et al., 2011) each harbouring multiple serovars, distinct variations found within the subspecies of a bacteria, and are usually classified based on the surface antigens they possess (Tindall et al., 2005). *Salmonella* has evolved to have a long standing association with its host, especially in vertebrates. Biological   niches for these bacteria include gastrointestinal tracts of pigs (Boyen et al., 2008), rodents (Valdezate et al., 2007) and poultry (Gast, 2007) as well as in amphibians such as frogs (http://www.cdc.gov/salmonella/water-frogs-0411/, 2011).

1.6.1 Route of Infection

Naturally, *Salmonella* is acquired when food or water containing the bacteria is ingested orally or through contact with an infected carrier. *Salmonella* is equipped with an adaptive acid tolerance response that is vital for its survival in the stomach (Muller et al., 2009). A portion of the infecting dose survives the low pH in the stomach and is transported to the small intestine where it forms colonies and establishes an infection. *Salmonella* encodes a ShdA protein that enhances its ability to colonise the intestine (Kingsley et al., 2003). Microfold (M) cells are located within the follicle-associated epithelium (FAE) of the Peyer's patch (PP). They are preferentially exploited by *Salmonella*, and are the primary sites of infection. The M

cells sample the gut for antigens which they transport to lymphoid tissues (Jepson and Clark, 2001) (Figure1.5). In the lymphoid tissue, *Salmonella* is taken up by dendritic cells (Wick, 2002) and other cells expressing CD11c for processing and presentation to immune cells. (Tam et al., 2008).

Figure 1.5 Diagrammatic representation of *Salmonella* entry into the host
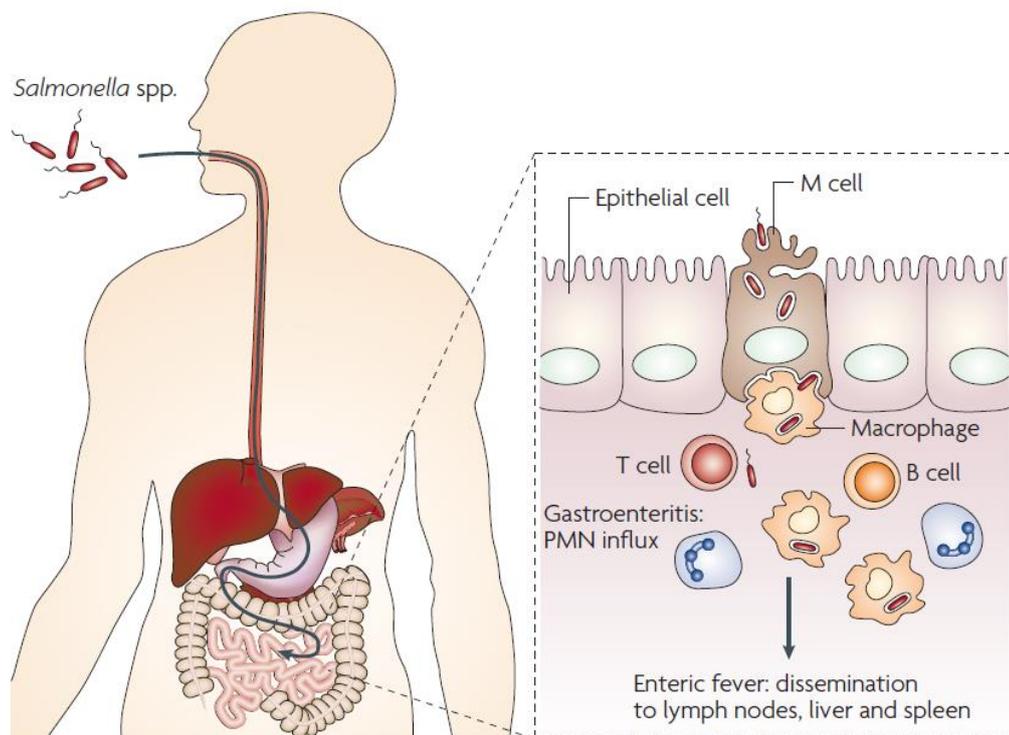
The diagram illustrates the entry of *Salmonella* into M cells and that survival in macrophages is a key step in the dissemination of *Salmonella* in the host (Haraga et al., 2008).

1.6.2 Pathogenicity of *Salmonella*.

Generally, *Salmonella* are classified as invasive and intracellular bacteria, infecting immune cells such as neutrophils, macrophages and dendritic cells to survive and proliferate within them. These microbes have the ability to modify membrane bound

compartments within the cells they infect (especially macrophages), creating for themselves a niche known as '*Salmonella*-containing vacuole' (SCV) (Szeto et al., 2009). To live successfully within the SCV, *Salmonella* encodes multiple systems that enable it to interact with the mucosal epithelia and penetrate through this barrier. Virulence of *Salmonella* is encoded in genes harboured in a genomic locus called *Salmonella* Pathogenicity Island (SPI). These genes are also associated with survival and fitness of the bacteria, with guanine-cytosine (GC) content different from the rest of the genome (Wisner et al., 2012). In *Salmonella*, about 21 SPI have been identified. Within the SPI are the type three secretion systems (TTSS) that are responsible for *Salmonella* invasiveness and persistence. The SPI-1 TTSS transfers effector proteins into the host cells. These proteins induce physiological and structural changes that enable the bacteria to be taken up into the host cells. Within the cells, *Salmonella* then expresses the SPI-2 TTSS. The SPI-2 TTSS regulates the internal SCV processes and interactions between the SCV and endosomal trafficking processes (Guiney and Fierer, 2011).

Salmonella enters the host by two mechanisms. The first mechanism is through phagocytic uptake by phagocytic cells such as macrophages, while the second involves SPI-1 TTSS action on non-phagocytic cells. When *Salmonella* is bound to epithelial cells, *Salmonella* secretes effector proteins into the host's cell triggering membrane ruffling and inducing the reorganisation of the cell cytoskeleton (Figure 1.6). Once within the epithelial cells, these effector proteins switch off and the cells are returned to their original state. The trapped bacteria are encased within the SCV. Unlike many other intracellular pathogens that leave the vacuolar space and migrate

to the cell cytoplasm, *Salmonella* remains within the SCV (Ibarra and Steele-Mortimer, 2009, Wisner et al., 2012, Bhavsar et al., 2007).

Toll like receptors (TLR) recognise *Salmonella* and trigger the appropriate immune response. As shown by Arpaia et al., (2011) activation of TLR 2, 4 and 9 contributes to acidification of the SCV leading to increased bacterial replication. (Arpaia et al., 2011). This reduced acidity and hindered SPI-2 induction, results in 3 major consequences; bacteria is released from the SCV into the macrophage cytoplasm, the vacuole fuses with the lysosome causing bacterial death, irregularly shaped bacteria and hence reduced bacterial survival (Buckner and Finlay, 2011). From their work, it can be argued that TLR signalling in *Salmonella* infection is more beneficial to the bacteria than to the host.

Figure 1.6 *Salmonella* induced membrane ruffling by SPI-1 TTSS effector protein is an important step in the invasion of *Salmonella* to host cells, Haraga et al., (2008)
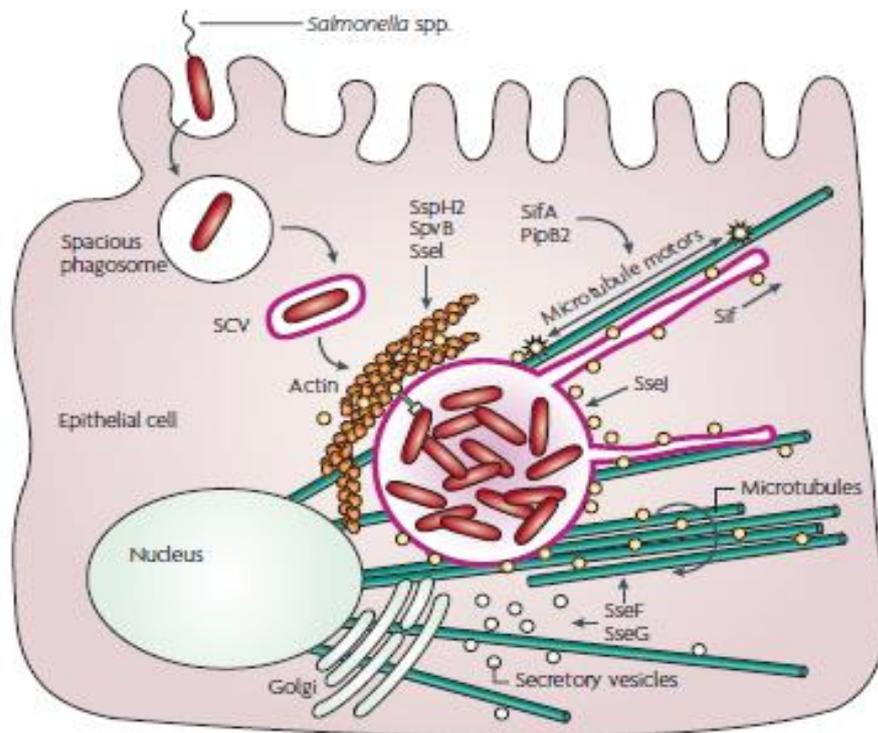
The diagram illustrates *Salmonella* induced ruffling of the cell membrane that faciliates entry of bacteria into the host cell. The internalised bacteria are located within the phagosome which fuses with the lysosome to form the *Salmonella* containing vacoule. Within the SCV, the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system (T3SS) secretes effector proteins such as SifA and PipB through the microtubules.

1.6.3 *Salmonella* Infections

In a review by Griffin and McSorley, *Salmonella* infections were broadly classified under three categories:

- Localised intestinal infection otherwise known as gastroenteritis

- Systemic infection of an otherwise healthy host, also known as typhoid

- Systemic infection of an immunocompromised or susceptible host also known as non-typhoidal Salmonellosis (Griffin and McSorley, 2011).

Figure 1.7 *Salmonella* isolates in England and Wales, 2000-2012. From https://www.gov.uk/government/publications/salmonella-by-serotype/salmonella-by-serotype-2000-to-2010
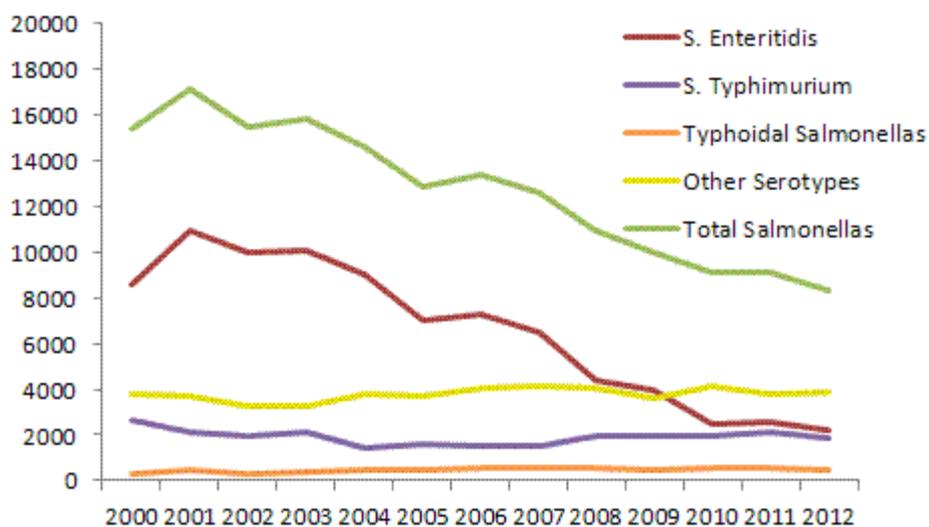
Salmonella infection results in various clinical syndromes (Figure 1.7). In fact, one isolate of this bacterium can lead to different clinical manifestations in different hosts (Table 1.3). The outcome of interaction between *Salmonella* and the host depends on a number of factors such as infecting dose, gut flora, host species and immunological competence of the host (Gordon, 2008). Figure 1.7 shows the number of reported cases of *Salmonella* isolates in England and Wales between 2000 and 2012. The figure also indicates that there has been a decrease in the number of *S. Enteritidis* isolates and total *Salmonella*. This is mainly due to decreased episodes of

Salmonella Enteritidis, as a result of greatly improved and monitored food safety and chicken breeding conditions (O'Brien, 2013).

Table 1.3 Salmonella infections

S. entrica serovar	Host	Salmonellosis	Symptoms and diseases
Typhi, Paratyphi	Human (restricted)	Typhoidal	Fever, enteric fever, abdominal pain, constipation, transient diarrhoea, maculopapular rash
Typhimurium, enteritidis	Broad range	Non-typhoidal	Gastroenteritis, abdominal pain, vomiting, inflammatory diarrhoea

1.6.4 Innate Immune Responses to *Salmonella*

The initial immune response to *Salmonella* infection is marked by the activation of phagocytic activities and production of inflammatory cytokines (Mäkelia and Hormaeche, 1997). Macrophages and neutrophils are essential at this stage of the infection in that they control the growth of bacteria by exerting both bactericidal and bacteriostatic activities (Grant et al., 2008). Nitric oxide modulates anti-*Salmonella* activities at later stages of the infection. Mastroeni (2002), showed that both NADPH oxidase and inducible nitric oxide synthase (iNOS) are required for host resistance to *Salmonella* in experiments using iNOS-deficient and wild type mice challenged with avirulent LT2 strain of S.*enterica* serovar Typhimurium (Alam et al., 2002). iNOS mice succumbed to infection while wild type mice survived. Reactive oxygen intermediates (ROI) mediate clearance of *Salmonella*. ROI do not act directly on

intracellular *Salmonella* but induce a Cathelicidin-related antimicrobial peptide (CRAMP) (Mastroeni, 2002). At this early phase of an infection, sphingomyelinase, a constitutive acid in macrophages, mediates NADPH- oxidase killing of S*almonella* (McCollister et al., 2007). In chronic granulomatous disease, (CGD) where cells of the immune system are defective in their ability to form reactive oxygen species, patients are susceptible to periodic microbial infections such as Salmonellosis (Mouy, 1989). In genetically modified TNFα knockout mice, protective multicellular lesions do not properly formed, leading to the rapid spread of *Salmonella* within liver tissues suggesting that TNFα is a major requirement in S*almonella* infection (Everest et al., 1998).

Dendritic cells (DCs) respond to *Salmonella* infection by up-regulating major histocompatibility complex class II (MHC-II) expression, as well as expression of co-stimulatory molecules CD40, CD80 and CD86 (Kalupahana et al., 2005). These processes enhance the ability of DCs to present *Salmonella* antigens to CD4, and CD8 T cells and B cells, serving as a bridge between the innate and adaptive immune system in *Salmonella* infection (McSorley et al., 2002). Neutrophils check the spread of *Salmonella* from the point of infection to systemic tissues. Consequently, a higher risk of bacteraemia is observed in neutropenic patients during infection with NTS (Conlan, 1996).

On infection, macrophages secrete IL-12 and IL-18 that stimulate NK cells to produce IFNγ. Mice deficient in IFN-γ receptor or mice treated with anti-IFN-γ antibodies and challenged with a 5 x 10^5 dose of SL3261 strain of *Salmonella* were impaired in their ability to clear the bacteria and finally succumbed to infection (Mastroeni et al., 2000).

1.6.5 Adaptive Immune Responses to *Salmonella*.

Early phases of *Salmonella* infection lead to the activation of the innate immune response. Though successful at checking growth and spread of the bacteria at this stage, it does not offer full protection to the host and makes the adaptive immune response essential for fighting *Salmonella* infection (Hughes and Galan, 2002). This stage does not require T cell or B cell responses. In T-cell knockout mice as well as in CD 4$^+$ or CD 8$^+$ deficient mice, the immune system is still able to suppress growth and spread of *Salmonella* in its early phase of infection (Hess, 1996).

To achieve full and effective control of *Salmonella* infection, *Salmonella*-specific T-cell responses must be developed and recruited to sites of infection. Although B cells appear dispensable in the later phases of *Salmonella* infection, T-cells are needed for their maturation and isotype switching to produce antibodies specific for *Salmonella* cell wall antigens. Srinivasan and his team (2004) have suggested that T-cells offer protection to the host by attacking infected cells containing the SCV, while bacteria that have escaped through the cells to establish infection in different tissues are targeted by antibodies (Srinivasan et al., 2004). CD8$^+$ T cells that mature into cytotoxic T lymphocytes contribute to protection against *Salmonella* when they liberate these bacteria from infected macrophages by induction of apoptosis. T cells activate *Salmonella*-infected macrophages via IFNγ production leading to increased killing of intracellular bacteria. However, bacteria that escape from infected cells are targeted by antibodies, produced by B cells via Th2 cell help. Th17 T cells express the cytokines IL-17A, IL17-F and IL-22, which coordinate mucosal immune responses to invading *Salmonella* by activating the expression of chemokines and C-type lectins (Liu et al., 2009).

The effector responses of *Salmonella*-specific CD4 T cells have been studied in both resistant and susceptible mice. Upon infection, these cells expand and acquire Th1-effector functions. These effector functions up-regulate their secretion of IL-2, IFN-γ and TNFα (Johanns et al., 2010). This expansion occurs within lymphoid tissue (see Figure 1.8), after which activated CD4 T cells acquire the ability to home to sites of infection. Stimulation of activated *Salmonella*-specific CD4 T cells occurs either through ligation of T cell receptors (TCR) or is initiated by microbe associated molecular patterns (MAMPs) such as bacterial LPS. However, there are insufficient data to show whether this stimulation is achieved by recognition of PAMP directly by activated T cell or via inflammatory mediators IL-12, IL-18, IFN-γ and TNFα (Griffin and McSorley, 2011).

In HIV infection, where there are profound immunosuppressive effects on both T-cell and macrophage mediated immune response, the importance of T-cell mediated immunity is emphasised. Deficiency in these immune responses increases a patient's susceptibility to *Salmonella* infection (Celum, 1987).

S. Typhi infects humans and other higher primates, exclusively; to cause a systemic infection called typhoid fever. For this reason, there is no small animal model of infection with *S.* Typhi, instead *S.* Typhimurium infection in mice is used. *S.* Typhimurium causes a similar systemic ailment in mice, usually short-lived, superficial gastroenteritis. Data from human and mouse studies have shown similar immune response with a major dependence on IFNγ production to activate macrophages. In humans antibody production can provide long-lasting protection, a response that cannot be modelled in the mouse.

Figure 1.8 Expansion of CD4 T cells during *Salmonella* infection. From Griffin & McSorley, (2011). Inflammatory responses are a key tool in the immune system's anti-*Salmonella* responses. Most effector cells required in this response are activated by inflammatory cytokines.
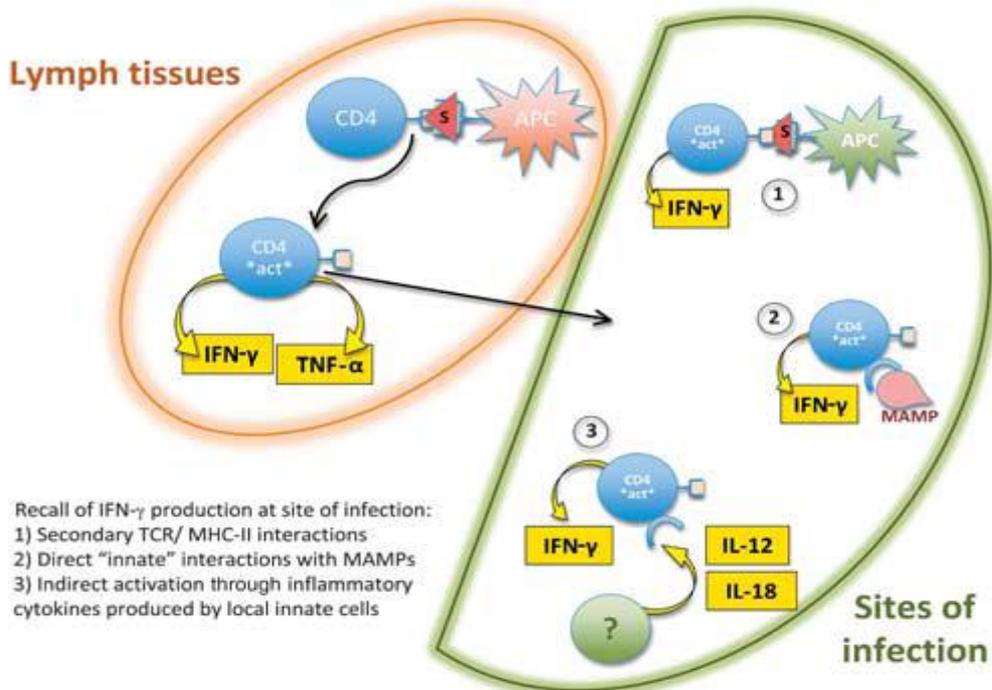
1.6.6 Resolution of *Salmonella* infection.

Resolution of *Salmonella* primary infection is mainly a combination of both innate and adaptive immune responses as shown in Figure 1.9. The B cells and antibody also contribute in controlling infection in both murine and human *Salmonella* infection. Antibody up-regulates the anti-microbial functions of reactive oxygen species and *Salmonella* uptake through Fc receptors (Menager et al., 2007). Mice lacking the micro RNA mir155 have impaired B cell functions and are poor producers of antibody against invading bacteria (Baltimore et al., 2008). Genetically engineered mir155 knockout mice show a defective protective response to *Salmonella* on both primary and secondary challenge of avirulent *Salmonella*. Apart from production of anti-

Salmonella antibodies, B cells to anti-*Salmonella* Th1 cell expansion, Th1 protective response to *S. enterica* is only partially developed when B cells are absent (Barr et al., 2010).

Figure 1.9 A brief diagrammatic representation of arms of the innate and adaptive immune response involved in response to *Salmonella*.
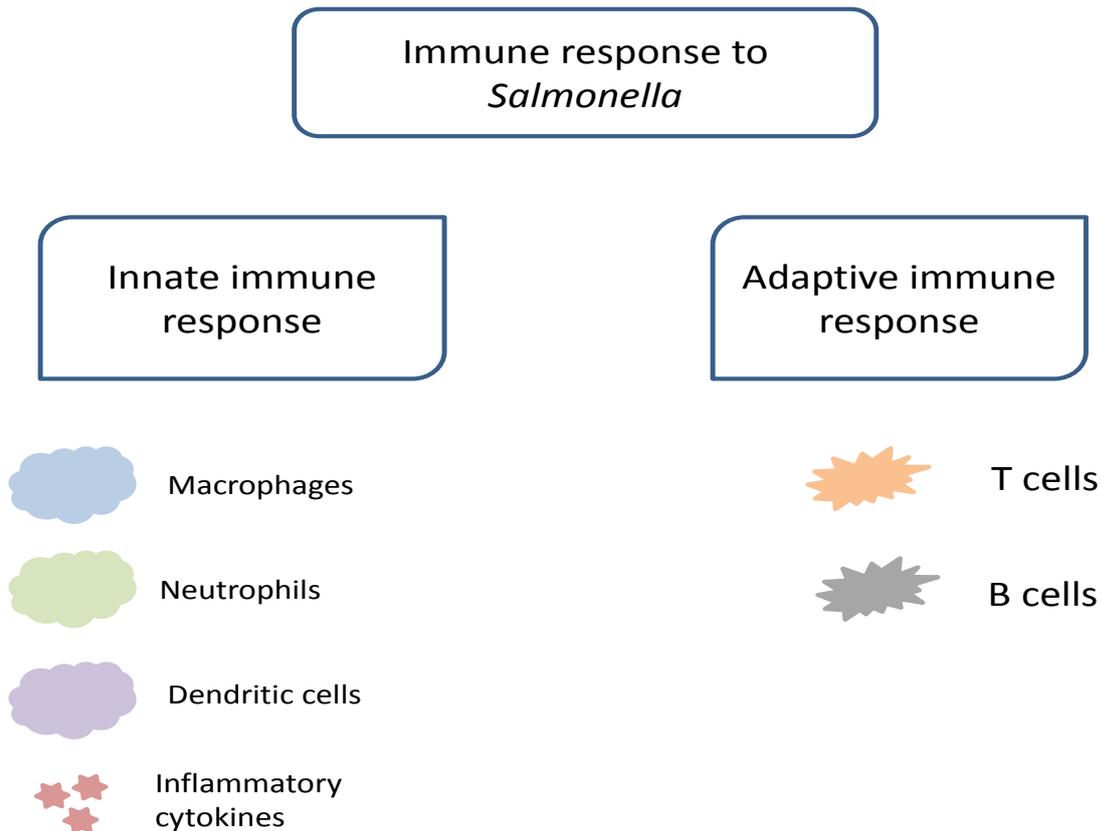
Immunodeficient disorders in humans have also been associated with a high risk of *Salmonella* infection. In individuals with X-linked agammaglobulinemia, where mutations in tyrosine kinase Btk profoundly impair B cell development, persistent recurrence of diarrhoea caused by *Salmonella* was reported (Kaku et al., 2002). Common variable immunodeficiency (CVID), characterised by abnormal cell mediated immunity and hypogammaglobulinemia, also predisposes sufferers to increased risk of *Salmonella* infections (Leen et al., 2006). In individuals with X-linked

hyper IgM syndrome, mutations occur in the gene coding the CD40 ligand expressed on activated T Cells. CD40 ligand on T cell binds to CD40 on B cells following contact with antigen, which induces B cell proliferation, immunoglobulin switching and antibody secretion (Wykes, 2003). IgA, IgE, and IgG levels in such individuals are markedly low but they have elevated IgM levels. These variant cells result in weak interactions between T cells and B cells. Isotype switching and generation of immune memory is compromised as a result. *Salmonella* infections have been described in a number of these patients (Levy et al., 1997).

1.7   Hypothesis

The hypothesis tested in this project was that during antimicrobial therapy, exposure to antibiotics impacts on the immune system, providing a beneficial response that will alter the immune response to bacterial infection, and ultimately, the outcome of such therapy.

1.8   Aim

To explore the above hypothesis, the aims of the project were to systematically examine the effect of antimicrobial drugs at concentrations mimicking the maximum serum concentrations (C_{max}) and minimum inhibitory concentrations (MIC) on;

- Bacteria – function and survival.
- The innate immune response of the host to bacteria.
- The interaction between bacteria and the host.

CHAPTER TWO

2   Materials and Methods

2.1   Bacterial Strains

This project used a variety of isogenic strains derived from wild type *S.* Typhimurium SL1344 (Table 2.1). The strains were stored on Protect™ beads (Technical Service Consultants Ltd., UK) at 20°C. Bacteria strains were resuscitated from beads on Luria-Bertani (LB) agar plates (Sigma-Aldrich Ltd, UK) and incubated at 37°C for 24 hours. The plates with bacterial colonies were stored at 4°C for two weeks. The strains were identity checked by gram stain and PCR. Overnight cultures of bacteria were grown by inoculating the appropriate volume of LB broth (Sigma-Aldrich, UK) with a single colony and incubating overnight at 37°C in a shaking incubator (200rpm).

2.2   Effect of antibiotics on viability of bacteria growing *in vitro* in liquid culture

The antibiotics used in this study were chosen as they are commonly used to treat infections and represent different classes of drug. To determine the effect of the antibiotics on bacterial viability in the absence of immune cells, bacterial strains were grown overnight in 10 ml of LB broth at 37°C with shaking. From the overnight culture, 200µl was sub-cultured into 10 ml of fresh LB broth and incubated with shaking at 37°C until the OD675 was approximately 0.2-0.3. This gave approximately 1 x 10^8 CFU/ml.

Appropriate volume of stock antibiotics corresponding to the desired concentration needed for the experiment was added to the bacteria culture. A 100µl aliquot was removed and denoted as T_0. The bacteria cultures were further incubated at 37°C

with shaking. Further aliquots of bacterial suspension were taken after 30 and 60 minutes. The viable count was determined by serially diluting the aliquot of the bacterial suspension in sterile distilled water. Twenty microlitres of each of the dilution sub-cultured to LB agar plates and incubated overnight at 37°C overnight. The number of colonies was counted afterward and colony forming units of the original suspension was determined.

2.3   Effect of antibiotics on the functional activities of human neutrophils

2.3.1 Subjects

Volunteers for this study comprised healthy individuals between the ages of 24 and 34, who were not under any medication. Venous blood was collected using the Vacutainer™ system of blood collection into lithium heparin tubes (Grenier Bio-one UK). Two tests were carried out because I investigated two key neutrophil defence mechanisms against bacterial infections. Phagotest quantifies neutrophil phagocytosis by determining the percentage of phagocytes which ingest fluorescently labelled bacteria, while phagoburst quantifies neutrophil oxidative burst by determining the percentage of neutrophils which oxidise the fluorogenic substrate dihydrohodamine (DHR) 123. (Hirt et al., 1994).

Table 2.1 *Salmonella* Typhimurium strains

Lab code	Genotype	Relevant characteristics	Description	Reference
L354	Wild type		Virulent strain	Wray and Sojka, 1978
L109	SL1344 tolC*::aph* transduced from a tolC*::aph* strain into a wild type background	Kanamycin resistant	Defective in efflux and is avirulent	Buckley *et al.,* 2006
L730	∆aroA from SL1344	Auxotrophic Kanamycin resistant	Defective in aromatic amino acid synthesis is avirulent in tissue culture and mice	(Hoiseth and Stocker, 1981)
L1449	SL1344 SPI-1*::aph*	Kanamycin resistant	The two ends of SPI1 locus is separated by an *aph* gene cassette.	Schechter et al., 1999

Note: the control strains SL1344 tolC*::aph*, SL1344 ∆aroA and SL1344 SPI-1*::aph* were only to distinguish invasion and phagocytosis and are not pathogenic strains of *Salmonella*. We concentrate on the virulent strain SL1344.

Table 2.2 The details of the exemplar antibiotics used in this study

Antibiotic	Concentration used[a]	Solvent	Source
Ciprofloxacin	0.5 µg/ml (Cacchillo and Walters, 2002)	SDW[b] + drop of acetic acid	Fluka BioChemika (17850)
Tetracycline	2 µg/ml (Agwuh and MacGowan, 2006)	SDW	Sigma (T3383)
Ceftriaxone	82 µg/ml (Patel et al.)	SDW	Sigma (C5793)
Azithromycin	2.93 µg/ml (Kroboth et al., 1982)	SDW + drop of EtOH	Sigma (E5389)
Streptomycin	30 µg/ml	SDW	Sigma S9137

[a]C_{max}: maximum concentration achieved in human serum
[b]SDW: sterile distilled water.

2.3.2   Neutrophil phagocytosis assay (Phagotest assay)

For the determination of the effect of antibiotics on the phagocytic activities of neutrophils, assays were carried out using PHAGOBURST® (10-0100) and PHAGOTEST® (10-0200) kits (Glycotope Biotechnology), following the instructions. Incubation period, temperature and volumes were strictly adhered to.

One hundred microlitres of heparinised whole blood were added directly to four 5ml polypropylene round-bottom FACs tubes (BD Biosciences). This was done neatly, ensuring that blood was not smeared on the side wall of the tubes.   Twenty microlitres of FITC labelled opsonised *E. coli* containing ~ 1 x 10^7 bacteria were added to tubes containing heparinised whole blood. The tubes were grouped into

two. The first set of tubes was incubated on ice, while the second set was incubated in a pre-warmed water bath set at 37°C, both for 10 minutes. The tubes incubated on ice served as the negative control.

After the 10 minutes incubation, the tubes incubated at 37°C were stacked on ice to stop phagocytosis. One hundred microlitres of ice-cold quenching solution was added to each tube and mixed by vortexing. The quenching solution suppresses the fluorescence of any bacteria not phagocytosed by the leukocytes. To each tube, 3 ml of ice-cold wash solution was added and centrifuged at 250 x g for 5 minute at 4°C, and the supernatant discarded. This washing step was repeated twice. Two millilitres of lysis solution at room temperature was added to each of the samples. The samples were vortexed and incubated at room temperature for 20 minutes. On return, samples were centrifuged at 250 x g for 5 minutes at 4°C and the supernatant discarded. The washing step was repeated as described above. For the DNA staining, 200 µl of DNA staining solution was added to each sample, vortexed and incubated on ice (in a light protected ice box) for up to 60 min.

Phagotest Flow cytometry analysis

Approximately 10,000 cells per sample were collected and analysed by fluorescence activated cell sorting using blue-green excitation light (488nm argon-ion laser).

Phagotest Data acquisition

Flow cytometry data was acquired by CyAn™ ADP Analyzer and analysed using Summit software V4.3. Neutrophils were identified and gated on forward scatter (FSC) and side scatter (SSC) characteristics. The cell counts measured the percentage of neutrophils that had phagocytosed *E. coli*. The mean fluorescence

intensity (MFI), i.e. the number of bacteria taken up per cell, was taken into account and used to calculate the phagocytic index. The data used in this analysis is expressed as the neutrophil phagocytic index.

For calculation of phagocytic index, the following formula was applied:

$$\text{Phagocytic index} = \frac{\% \text{ of cells with ingested bacteria} \times \text{MFI}}{100}$$

2.3.3 Neutrophil oxidative burst assay (Phagoburst Assay)

One hundred microlitres of heparinised whole blood were carefully dispensed directly to the bottom of 5ml polypropylene round-bottom FACs tube (BD Biosciences). The tubes were grouped into four sets. One set each for the four different stimuli was tested; wash solution, *E. coli* bacteria, N-formylmethionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA). Each set was done in triplicate.

To activate the assay, 20 µl of wash solution was pipetted into the first set of tubes containing blood samples. This served as the negative control. 20 µl of pre-cooled, vortexed *E. coli* bacteria was pipetted into the second set of tubes and this was the test sample. 20 µl of fMLP working solution was added to the third set of tubes, which served as the low stimulus while 20 µl of PMA working solution was added into set 4 which served as high stimulus. The samples were vortexed and incubated at 37°C for 10 minutes in a pre-heated water bath with lid.

After 10 minutes of incubation, 20 µl of substrate solution was added to each tube and vortex thoroughly. The samples were incubated at 37°C for another 10 minutes.

To each samples, 2ml of lysis solution (at room temperature) was added. The samples were vortexed and incubated at room temperature for 20 minutes. On return samples were centrifuged at 250 x g for 5 minutes at 4°C and the supernatant discarded. The samples were washed by adding 3 ml of ice-cold wash solution, vortexed and centrifuged as above and the supernatant discarded. As with the Phagotest assay, 200 µl of DNA staining solution was added to each sample, vortexed and incubated for 10 minutes on ice (in a light protected ice box) for not longer than 60 minutes.

Phagoburst Flow cytometry analysis

Approximately 10,000 cells per sample was collected and analysed by fluorescence activated cell sorting using blue-green excitation light (488nm argon-jon laser).

Phagoburst Data acquisition

Flow cytometry data was acquired by CyAn™ ADP Analyser and analysed using Summit software V4.3. Live gate was set on the neutrophil cluster on the FCS vs. SSC. Reactive oxygen production by neutrophils as well as mean fluorescence intensity was analysed.

2.4 Effect of antibiotics on killing of opsonised *S.* Typhimurium SL1344 wild type (L354), *tolC::aph* (L109) and *aroA::aph* (L730) by neutrophils.

2.4.1 Neutrophil Isolation

Two per cent dextran was added to heparinized whole blood at a ratio of 1ml of 2% dextran to 6ml blood. The mixture was given a gentle mix and incubated at room temperature for 30 minutes. After incubation, 5ml of a 56% Percoll® solution was added to 15ml falcon tubes. Using a fine tipped Pasteur pipette, 2.5 ml of 80%

Percoll® solution was then carefully layered underneath the 56% Percoll®, making sure that bubbles were avoided, and the 56% Percoll® layer was not disrupted. At this point the red blood cells had sedimented in the dextran-blood mixture leaving a 'buffy coat' top layer containing the white blood cells. The buffy coat was aspirated from the mixture and carefully layered above the 56% Percoll®. The sample was centrifuged at 1100 rpm for 25 minutes at 5°C.

After centrifugation, a layer of PBMCs appeared between the buffy coat and 56% Percoll®, while the layer of neutrophils appeared between the 56% Percoll® and the 80% Percoll® layers. The layer containing neutrophils was pipetted carefully in a circular manner using a fine tipped sterile Pasteur pipette and washed in 10 ml of HBSS. Care was taken not to disrupt the layers during pipetting or contaminate the desired cells with infiltrates from the surrounding media. The isolated neutrophils were centrifuged at 1600 rpm for 10 minutes, re-suspended in HBSS and counted. Cells were used within 4 hours of collection. The isolated neutrophils were suspended in HBSS containing appropriate concentrations of antibiotics and incubated with opsonized bacteria at 37°C.

2.4.2 Preparation of 20% human serum

Blood was collected into an anti-coagulant free sterile tube and allowed to clot for 30 min. The clotted blood was centrifuged at 3000 rpm for 10 minutes. The supernatant (serum) was aspirate using a Pasteur pipette into a sterile tube. Appropriate volume of HBSS was added to give a solution containing 20% human serum.

2.4.3 Opsonisation of S. Typhimurium

Overnight culture of *S.* Typhimurium grown in LB broth was centrifuged at 2200 rpm for 15 minutes. The pelleted cells were re-suspended in 10 ml sterile DPBS and

centrifuged as above. This was repeated twice to wash off any residual media. Opsonisation was carried out by incubating the bacteria in the prepared 20% human serum for 30 minutes. After opsonisation, the cells were harvested and washed in 10 ml sterile PBS.

For the killing assay, antibiotic treated or untreated neutrophils were incubated with the opsonized *S.* Typhimurium at 37°C. At 0, 30 and 60 minutes, an aliquot of the sample was taken out, lysed in sterile distilled water and plated out on LB agar plates. The plates were then incubated at 37°C for 24 hours in 5% CO_2. Single colonies that grew on the plates were counted. Statistical analysis was carried out using Excel for Windows.

2.5   J774 tissue culture

2.5.1 Resurrection of cells from liquid nitrogen and culture of monolayers

J774.6 murine macrophages were previously stored in liquid nitrogen. A vial containing cells was removed from liquid nitrogen and the cells thawed quickly by incubation in a water bath at 37°C. The outside of the vial was cleaned with 70% ethanol and the suspension was transferred to a universal tube inside a biosafety cabinet. Ten millilitres of complete DMEM medium (Sigma UK, D5671) was added drop wise to the cells and swirled carefully to mix. Complete tissue culture medium (DMEM) comprises Dulbecco's modified eagle medium containing 1% (v/v) non-essential amino acids (NEAA) (Sigma UK, M7145), 1% (v/v) L-glutamine (Sigma UK, G7513) and 10% (v/v) fecal serum. The cells were then harvested by centrifugation at 1500 x g for 5 minutes. The supernatant was discarded and cells re-suspended in 25ml of complete DMEM medium. The cell suspension was transferred to a 75cm^2

flask (Corning®) and incubated in a 37°C humidified incubator until cells formed a monolayer and became confluent when viewed under an inverted microscope. Association and invasion assays were performed in 24 well plates. Each well of the plates were seeded with approximately 1 x 10^5 cells and incubated at 37°C with 5% CO_2 for 48 hours. To prepare the monolayers for the assay, each well was washed with Hank's Balanced Salt Solution (HBSS) by pipetting 1ml of HBSS to each well of the 24 well and removal three times to remove any floating cells or residual nutrient depleted medium. Association and invasion assays were carried out as described in Sections 2.8.2 and 2.8.3.

To resurrect the THP-1 cells, a vial from liquid nitrogen was thawed by incubation in water bath at 37°C. The thawed cell suspension was transferred to 10 ml of pre-warmed RPMI medium and harvested by centrifugation. The THP-1 cells were re-suspended in 15 ml media and incubated at 37°C with 5% CO_2 for 48 hours. Subsequently, the cells were activated by suspending in media containing 5 ng/ml of PMA (Sigma UK, P8139) at a concentration of 10^5 cells per ml. For association and invasion assays, 1 ml of the cells was added to individual wells of a 24 well plate and incubated as above for 2-3 days until they had adhered to the bottom of the wells. Association and invasion assays were carried out as described in Sections 2.8.2 and 2.8.3.

2.6   Isolation and purification of human monocytes using Ficoll-paque and CD14 MicroBeads

2.6.1   Sample collection

50 ml peripheral human blood was collected from healthy volunteers using the Vacutainer™ system of blood collection into lithium heparin tubes. Alternatively, blood was ordered from the Birmingham National Blood Service.

2.6.2   Ficoll gradient isolation

Human monocytes were isolated from peripheral blood by Ficoll density gradient centrifugation. The blood sample was diluted 1:1 with sterile PBS and mixed gently in 50 ml Falcon™ tubes. Fifteen millilitres Ficoll was added to 50 ml Falcon tubes. Twenty-five millilitre of the diluted blood was layered over the Ficoll layer, carefully not to disrupt the interface between the blood and the Ficoll layer. This was centrifuged for 30 minutes at 400 x g at room temperature and zero deceleration. After centrifugation the blood was separated into three distinct layers based on cell density; a layer of clear fluid above (the plasma), a layer of red blood cells below and a thin whitish layer in between containing the white blood cells known as the "buffy coat".

The buffy coat layer was carefully collected using a Pasteur pipette into universals containing 10 ml RPMI (serum free). The cells were washed with serum-free RPMI to get rid of the Ficoll by centrifugation for 8 minutes at 400 x g at room temperature. The cells were re-suspended in 8 ml of RPMI and centrifuged again as above.

The counting chamber of the haemocytometer and coverslip were cleaned. Ten microlitres of the cell suspension was loaded onto the sample introduction point (at

the top of the coverslip) and the cell suspension was taken up by capillary action. The counting chamber was then placed on the microscope stage and the counting grid bought into focus with the 4x objective. The cells in each square were then systematically counted. The cells suspension was centrifuged again as above and the media decanted.

2.6.3 CD14$^+$ monocyte isolation.

This method is based on the magnetic-activated cell sorting (MACS) technique developed by Miltenyi Biotech. For isolation of CD14$^+$, the isolated monocytes were re-suspended in MACS buffer (1 x PBS + 0.5 % BSA + 2 mM EDTA) at a ratio of 80µl of MACs buffer per 10^7 cells. CD14 MACs MicroBeads (Miltenyi Biotec) were added at a ratio of 20ul of bead suspension per 10^7 cells. This was then incubated in the fridge for 15 minutes to allow the CD14 expressing monocytes to bind to the beads.

Five millilitre of MACs buffer was added to the cell-bead suspension and washed by centrifugation for 8 minutes at 400 x g at room temperature. The resultant pellet was re-suspended in 500ml MACs buffer per 10^8 cells. The LS column adapter (Miltenyi Biotec) was inserted into the MidiMACS^{TM} magnetic separator (Miltenyi Biotec). The column was primed by running 3 ml of MACs buffer through the column. The cell suspension was run through the column and unbound cells were collected in a universal. The column was washed three times with 3 ml MACs buffer and collected in a universal tube.

To collect the bound CD14$^+$ monocytes, the column was taken off the magnet and 5 ml MACS was pipetted into the column. The contents of the column were expelled by firmly pushing the plunger (provided in the adaptor pack) into the column. The CD14$^+$ monocytes were centrifuged for 8 minutes at 400 x g at room temperature.

The cells were counted and re-suspend at a density of 5×10^5 per ml in RPMI. One millilitre of the cell suspension was seeded to individual wells of a 24 well plate.

2.6.4 Generation of M1 macrophages from CD14⁺ monocytes

To generate M1 macrophages, GM-CSF (Life Technologies, PHC 2015) was added to the cell suspension to give a final concentration of 10 ng/ml respectively. One millilitre of the cell suspension was added to individual wells of a 24 well plate and incubated at 37°C with 5% CO_2 for 72 hours in a humidified atmosphere. Post 72 hours incubation, the media was removed and replaced with fresh pre-warmed media containing 10 ng/ml of GM-CSF. The cells were incubated for a further 72 hours at 37°C and 5% CO2 in a humidified atmosphere.

2.7 Mouse bone marrow derived macrophages (BMDM).

Mouse tissue was obtained courtesy of Dr. Dalya Soond, Rheumatology Research Group, University of Birmingham. The donor mouse strain, C57BL/6J was used in all the experiments, aseptically handled, typically 10-12 months old. Using a pair of forceps and scalpel, muscles were detached from the femur and tibia to expose the bones. The bones were then cut at both epiphyses to create hollow tubing. To collect the bone marrow, the bones were placed in a 0.75 ml Eppendorf tube punctured at the bottom and inserted into a 2 ml Eppendorf tube. This was centrifuged in a bench centrifuge at 4 rpm for 3 minutes. The centrifugal force pushed the bone marrow through the hollow part of the bone into the Eppendorf tube. The bone marrow was re-suspended in 1 ml of complete media and made up to 50 ml in a Falcon tube. For differentiation to macrophages and adherence to tissue culture plates, M-CSF was added to the cell suspension to give a final concentration of 10ng/ml in 50ml. Ten

millilitres of the cell suspension was added to 90 mm tissue culture dishes and incubated at 37°C with 5% CO_2 for 6 days.

After incubation, the media was removed and the cells were washed twice with sterile pre-warmed PBS. To detach the adherent cells, 5 ml of pre-warmed non- enzymatic cell dissociation solution was added to the tissue culture plates and incubated at 37°C with 5% CO_2 for 15 minutes. Cells were lifted from the dish with a cell lifter and transferred to equal volume of pre-warmed fresh media. The cells were centrifuged at 400 x g for 6 minutes. The cells were then re-suspended in media at a density of 5 x 10^5 per ml, and 1 ml aliquots were added to individual wells of a 24 well plates. The plates were incubated at 37°C with 5% CO_2 for 24 hours, for infection assay the next day.

Both preparations for human M1 and murine bone marrow derived macrophages are extensively used and have been validated by flow cytometry in the laboratories of Piddock and Wallace. However, as this was not validated in the experiments described in this thesis it is possible that other cell types could have had an impact (Section 4.5 and 5.4).

2.7.1 Cell viability assessment

The trypan blue exclusion method was used. Ten microlitres of trypan blue (0.4% w/v) (Sigma, T8154) was mixed with 10 µl cell suspension cell suspension before inoculation with bacteria. This was left to stand for 2 minutes. The suspension was placed in a haemocytometer and viewed with a light microscope using an x10 lens. Clear cells were counted as viable, whereas cells stained blue were counted as non-viable.

2.8   Tissue culture infection assays

2.8.1   Preparation of bacteria for infection assays.

The bacterial strains were grown in LB medium overnight at 37°C with shaking. The bacterial cells were harvested by centrifugation at 2200 x g in a universal tube for 10 min at room temperature. The supernatant was discarded. The harvested cells were washed by re-suspending in 10 ml sterile phosphate buffered saline and re-centrifuged as before. This was repeated three times to remove any residual broth. The cells were re-suspended in 5ml PBS once again and adjusted to an optical density of 1.23 at 540nm by addition of sterile PBS. This suspension contained approximately 1×10^7 CFU/ml of bacteria. This suspension was then diluted 1:20 by adding 0.5ml of bacteria solution to 9.5ml of inoculation media. Inoculation media consisted of DMEM supplemented with 1%NEAA (Sigma, UK) and 1% L-glutamine (Sigma, UK).

2.8.2   Association assay

This assay was carried out to determine the number of bacteria associated with the monocyte monolayer. One millilitre of the bacterial suspension was added to each of the wells of the 24 well plate and incubated at 37°C with 5% CO_2 for two hours. The supernatant was carefully removed with a pipette and the monolayers washed with pre-warmed HBSS six times to remove the non-adherent bacteria. One milliliter of 1% (v/v) Triton-X 100 and a sterile magnetic flea was added to each well. The 24 well plate was placed on a magnetic stirrer for 10 minutes to disrupt the monolayer. From the disrupted monolayer in each well of the 24 well plate, a series of 1:10 dilutions were made. Three 20 µl drops of each dilution were sub-cultured to LB agar plates and the plates incubated at 37°C overnight. The dilutions that had growth of separate

colonies were identified and colonies counted. This was used to determine the number of colony forming units per millilitre.

2.8.3 Invasion assay.

This assay was carried out to quantify the number of bacteria that invaded the eukaryotic cell layer. To the eukaryotic cell layer, 1 ml of the already diluted bacterial suspension was incubated at 37°C in 5% CO_2 for two hours. The supernatant was removed and the monolayers washed three times with pre-warmed HBSS. The 24-well plate was gently tapped over an absorbent paper to blot away any residual media. To ensure that all the external bacteria were killed, 2 ml of inoculation media containing 100µg/ml gentamycin was added to each well and incubated at 37°C with 5% CO_2 for   two hours. Gentamicin does not damage or enter eukaryotic cells hence any intracellular bacteria are unharmed, while bacteria remaining outside the macrophage cells were killed, as judged by lack of colony formation. The antibiotic media was removed. The monolayers were then washed six times with HBSS. One millilitre of 1% (v/v) Triton-X100 and a sterile magnetic flea was added to each well and placed on a magnetic stirrer for 10 min to disrupt the monolayer. From the disrupted monolayer in each well of the 24 well plate, a series 1:10 dilutions was made. Three 20 µl drops of each dilution were sub-cultured to agar plates and the plates incubated at 37°C overnight. The dilutions that had growth of separate colonies were identified and colonies counted. This was used to determine the number of colony forming units per millilitres as previous paragraph.

Each association and invasion assay was carried out with a minimum of three biological and three technical replicates giving 9 data points for each experiment.

Mean CFU/ml and standard deviation were calculated for each biological replicate using Excel software. Graphs were plotted with error bars ± one standard deviation.

Adhesion level is calculated as the difference between the mean CFU/ml for association and the mean CFU/ml for invasion. The value and graphs were calculated and plotted on Excel software, (Windows, 2007). Graphs were plotted with error bars ± one standard deviation.

Comparison of adhesion and invasion of each strain of the bacteria to the wild type staring was carried out using a Student's T-test. Also the Student's T-test was used to analyze the differences between the results for invasion and adhesion for antibiotic treated and untreated macrophages. This was calculated on Excel software using the t-test function. Statistically significant values were taken at $P \leq 0.05$.

2.8.4 Effect of antibiotics on the adhesion to and invasion of bacteria in macrophage monolayers.

To explore the effect of prior exposure to antibiotic upon adhesion and invasion of bacteria in the J774.6 macrophage confluent monolayers, the cell culture supernatant was removed from the eukaryotic cells which had been incubated for 48 hours at 37°C in 5% CO_2. The monolayers were washed three times with 2 ml HBSS. This was replaced by 2ml of fresh complete growth media containing the desired concentration of antibiotic. The cell culture was further incubated for 2 hours. The 2 hour incubation period was chosen to mimic the time at which the maximum concentration (C_{max}) of the antibiotics in this study is achieved in volunteer studies (Shah et al., 1999, Rebuelto et al., 2002, Agwuh and MacGowan, 2006, Liu et al., 2011) and to distinguish the effects on the immune cells from the antibacterial treatment by the antibiotics. Post incubation, the macrophage monolayer was

washed three times with 2 ml of HBSS. The antibiotic-containing medium was then replaced with fresh medium. Procedures in section 2.8.2 and 2.8.3 were then followed for both association and invasion assays respectively.

2.9   TLR RT-PCR array

The RT^2 Profiler PCR array was used to analyse the expression of a panel of 84 genes central to the TLR signalling pathway of J774 murine macrophages. This profiling technology constitutes a set of optimised real time PCR array primer assays on a 96 well plate, to which cDNA from the samples are added, and then run on a real-time PCR array cycling program.

2.9.1 Isolation of RNA from antibiotic treated macrophages.

RNA was isolated using SV Total RNA Isolation System kit from Promega (Z3100, Promega). Macrophages were cultured and seeded on to 24 well plates as described above (2.5.1). To lyse the cells, 175 µl of RNA lysis buffer was added to each of the wells. This solution was pipetted up and down to mix, then transferred to a 1.5 ml RNase-free microfuge tube (AM12400, Ambion). To each microfuge tube 350 µl of RNA dilution buffer was added and mixed by gentle pipetting. This was placed in a heat block for 3 minutes and centrifuged at 13,000 x g for 10 minutes on a bench top centrifuge. Two hundred microlitres of 95% ethanol was added to the cleared lysate and mixed by gentle pipetting. The solution was transferred to the spin column assembly provided in the kit. This was centrifuged at 13,000 x g for 1 minute. The liquid in the collection tube of the spin column assembly was discarded. Six hundred microlitres of RNA wash solution was added to the columns and centrifuged as above. The DNase incubation mix was made up as described in the kit protocol. Fifty microlitres of the DNase incubation mix was added directly onto the membrane in the

spin column and incubated at 25 for 15 minutes. Two hundred microlitres of DNase stop solution was added to the spin column to stop the DNase activity. This was centrifuged at 13,000 x g for 1 min. The collection tube was emptied again. Six hundred microlitres of RNA wash solution was added to the column and centrifuged as above. Again, 250 of RNA wash solution was added to the column and centrifuged as before. The spin compartment was transferred to an elution tube. One hundred microlitres of nuclease free water was added to and centrifuged as above. The elution tube containing purified RNA was stored at -80 until the samples were measured for RNA and DNA content.

2.9.2 Determination of RNA concentration and purity by UV spectrophotometry.

The RNA concentration of the samples was measured using a Nanodrop ND-1000 spectrophotometer. One microlitre of the RNA sample was loaded on the sample pedestal of the Nanodrop and absorbance measurement was taken at 260nm and 280 nm. For assessment of purity, samples returning an $A_{260}:A_{280}$ ratio value of ~2.0 and an $A_{260}:A_{230}$ ratio value of >1.7 were regarded as pure and used for RT-PCR experiments.

2.9.3 Assessment of ribosomal band integrity.

Ribosomal band integrity was assessed by running an aliquot of each of the RNA samples through an agarose gel. Agarose gels were prepared by adding 1 g of electrophoresis grade agarose (check) to 100 ml of 1% Tris Boric acid-EDTA (TBE). This suspension was heated in a 650 W microwave for 2 minutes to facilitate the dissolution of the agarose and left to cool. Midori Green (Nippon Genetics, Germany) was added to the molten agarose to give a final concentration of 0.1 µg/ml. the

solution was poured into a gel tray and allowed to set. The gel was immersed in 1% TBE in the gel tank. A loading buffer was diluted 1:5 by adding 2 µl to 8 µl of RNA sample. The resulting 10 µl was loaded unto the gel along with a DNA 1kb sizing ladder (Bioline). Electrophoresis was used to separate the proteins in the samples at 100 volts for one hour. The quality of the 18s and 28s ribosomal RNA was visualised using gel images using Gene Tools (Syngene, U.K.).

2.9.4 cDNA synthesis from RNA samples.

cDNA was synthesized from RNA samples using RT^2 HT Strand Kit following the manufacturers' instructions. Buffers GE2 and BC4 transcriptase were removed from the -20°C storage and thawed on ice. Six microlitres of Buffer GE2 was added to nuclease free microfuge tubes. An 8 µl aliquot of the sample containing 0.5 µg total RNA was added to the Buffer GE2 in the microfuge tubes and gently mixed by pipetting up and down. The tubes were centrifuged at 1000 x g for 1 minute and incubated at room temperature for 10 minutes. After incubation, 6 µl of the BC4 Reverse Transcriptase Mix was added to the tubes and centrifuged at 1000 x g for 1 minute. The reverse transcription reaction was performed in a thermal cycler under the following conditions: 42°C for 15minutes, 95 °C for 5 minutes 4°C hold. The cDNA samples were stored at -20°C until required for RT-PCR.

2.9.5 Analysis of gene expression using RT-PCR.

The expression levels of genes in mouse macrophages in response to antibiotics or *Salmonella* was determined using RT-PCR. The cDNA template form reverse transcription was removed from the -20 C and thawed on ice. For each sample, the 20 µl cDNA was transferred to new tubes and re-suspended in 91 µl of RNase-free water. One hundred and two microlitres of this cDNA solution was added to 1350 µl

of 2x RT^2 SYBR Green Mastermix (SABiosciences, 330502) and 1248 µl of RNase-free water to make the PCR components mix in a loading reservoir (SABioscience, 338162). Twenty-five microlitres of the PCR components mix was dispensed into the Mouse Toll-Like Receptor Signalling Pathway RT-PCR array plate using an 8-channel multi pipette. The plate was sealed with an optical adhesive film and centrifuged at 1000 x g at room temperature to remove bubbles. The plate was placed on ice until the cycling conditions were set up in the Bio-Rad CFX96 real-time PCR cycler. The instruction for setting up the Bio-Rad CFX96 RT-PCR cycler was downloaded from www.SABiosceinces.com/pcrarrayprotocolfiles.php and strictly adhered to. The following cycling conditions were used; initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 minutes. The RT-PCR array plate was placed in the real-time cycler and transcription reaction was carried out.

2.9.6 Data analysis

The PCR reactions were performed in triplicate for each treatment group. Data analysis was carried out using the SABiosciences web based PCR array data analysis. The RT-PCR data in the form of cycle thresholds (CT) from the Bio-Rad software was exported to Microsoft Excel spread sheet. The CT value represents a point at which the fluorescent intensity generated in the PCR reaction reaches a set threshold above background signal. The SABiosciences data analysis website provided a template to which the CT data was transferred. The template was then uploaded on the website and the data was analysed using an integrated web based software package. Relative gene expression was calculated by ΔΔCT method

normalised against the CT values of 5 housekeeping genes (Actb, B2m, Gapdh, Gusb and Hsp90ab1) and expressed as fold change.

2.10 Determination of production of cytokines by J774 macrophages in the presence and absence of antibiotics using enzyme-linked immunosorbent assay (ELISA).

J774 macrophages were seeded onto 24 well tissue culture plates at a concentration of 1 x 10^5 cells per well and incubated at 37°C for 48 hours until they formed a monolayer. The media was removed and the monolayers were washed with HBSS three times to remove residual media. The macrophages were then incubated with fresh media alone for 2 hours or with media containing either C_{max} concentration of antibiotics (5 µg/ml of ciprofloxacin or 82 µg/ml of ceftriaxone), or wild type *S.* Typhimurium (SL1344). Another set of cells was incubated with antibiotics for 2 hours then washed and incubated with media containing SL1344. The cell culture supernatant were then collected in a 1.5 ml Eppendorf tube and stored at -20°C for analysis of cytokine content under the different conditions.

The Mouse TNFα (88-7064-22) and IL-1β (88-7013-22) Ready-Set-Go® kits (eBiosciences UK) were used to measure protein concentration of TNFα and IL-1β IL-6 by ELISA analysis. The kits were used in accordance with the manufacturer's specified protocol. Fifty microlitres of the capture antibody re-suspended in the Coating Buffer was added to each well of a Nunc Immuno™ 96 Microwell™ Plate. The plate was sealed and incubated overnight at 4°C. The next day, the contents of the wells were aspirated and washed three times with PBS Tween 20 (0.05%). The wells were then blocked with Assay Diluent and incubated for one hour at room temperature. The wells were washed again as above. Serial dilutions of the assay

standard were prepared to make a standard curve of 8 points. Fifty microlitres of the standard solutions were added to indicated wells in triplicates. Fifty microlitres of cell culture supernatant was also added to designated wells and incubated for 2 hours at room temperature. The wells were then aspirated and washed as above. Fifty microlitres of the detection antibody were added to the wells and incubated at room temperature for 1 hour. After incubation with detection antibody, the wells were aspirated and washed three times. Fifty microlitres of the detection antibody diluted in the Assay Diluent were added and incubated at room temperature for 30 minutes. The wells were aspirated and washed six times. Fifty microlitres of the substrate solution (tetramethylbenzidine, TMB) were added to the wells and left to incubate at room temperature for 15 minutes. The enzyme substrate reaction was stopped by adding 50 µl of stop solution (2N H_2SO_4) into each well. The plate was then read on a spectrophotometer (BioTek® Synergy HT, UK) using 450 nm as the primary wavelength within 5 minutes of adding the stop solution. The OD values for individual samples were calculated using a standard curve created by GraphPad Prism® software (GraphPad Software Limited, USA).

CHAPTER THREE

3 Effect of the maximum serum concentrations of antibiotics on *Salmonella* in liquid media (broth) *in vitro.*

3.1 Background

The therapeutic effect of antibiotics is dependent on administration schedules as well as the maximum available concentration of the drug attained in serum (C_{max}) during antibiotic therapy. For antibiotics to be effective at sites of infections they have to be present at concentrations above the minimum inhibitory concentration (MIC) or mutant prevention concentration (MPC) of the infecting pathogen (for susceptible pathogens) (Wise, 2003). MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism following an overnight incubation (Andrews, 2001), while MPC describes the concentration of an antimicrobial drug necessary to prevent emergence of isolates that have undergone first step mutation and acquired low level resistance (Boothe, 2006). However, administration of antibiotics is only one aspect of the elimination of bacteria from the host during an infection. In this chapter, the effect of clinically relevant concentrations of five antibiotics, (ciprofloxacin, ceftriaxone, tetracycline, azithromycin and streptomycin) alone on *Salmonella enterica* serovar Typhimurium growing in liquid media was determined. The above mentioned antibiotics are exemplar drugs, each representing a different class of antibacterial agents. More specifically, ciprofloxacin, ceftriaxone and azithromycin were chosen because they are used in the treatment of *Salmonella* infections (Sirinavin and Garner, 2000, Wong et al., 2014).

3.2   Hypothesis:

Antibiotics of different classes at C_{max} vary in their efficacy against *Salmonella* in liquid broth.

3.3   Aim:

To investigate the effect of five antibiotics, representing different classes, at clinically relevant concentrations, on the viability of *Salmonella* Typhimurium grown in liquid broth, and in the absence of the immune cells.

3.4   Results of experiments to investigate the effect of antibiotic concentration in liquid media (broth).

The effect of five antibiotics (ciprofloxacin, tetracycline, ceftriaxone, azithromycin, and streptomycin) was determined at different concentrations reflecting one-tenth of the MIC, the MIC and the C_{max} concentrations of each antibiotic. Viable counts were determined after 30 and 60 minutes of exposure to antibiotics. For these experiments, three strains of *Salmonella* Typhimurium were used; wild type SL1344 (L354), *tolC* mutant (SL1344 *tolC::aph*, L109) and *aroA* mutant (SL1344 Δ*aroA*, L730). The strains containing mutations in the *tolC* and *aroA* genes were chosen as controls for because they have previously been shown to be less virulent and less invasive than SL1344, making them suitable controls for use in subsequent tissue culture infection assays.

The MICs of two of the antibiotics (ciprofloxacin and tetracycline) against SL1344 (L354) and SL1344 *tolC::aph* (L109) was previously determined (Blair et al., 2009) and used for these assays. The remainder of the MIC were determined and detailed in Table 3.1. The C_{max} values for all five antibiotics have previously been published in the literature (Chapter 2, Table 2.2) and were replicated in these assays.

Table 3.1 MICs of the range of the five antibiotics against the three strains of bacteria used in these experiments.

	MIC µg/ml				
	Cip	Tet	Cef	Azi	Str
SL1344 (L354)	0.015	0.5	8	2	8
SL1344 *tolC::aph* (L109)	0.015	0.5	8	1	8
SL1344 *aroA∆* (L730)	0.015	0.5	8	2	8

When bacteria were exposed to 0.015 µg/ml of ciprofloxacin (MIC) there were no changes in the viability of *Salmonella* observed at either time points (Figure 3.1a). However when exposed to 5µg/ml (C_{max}) of ciprofloxacin after 30 minutes, there was three fold decrease in the number of bacterial colony forming units from all three strains. After 60 minutes antibiotic exposure, there was a > 4-log reduction in viable counts of both SL1344 *tolC::aph* (L109) and SL1344 ∆*aroA* (L730). When exposed to 5 µg/ml of ciprofloxacin, the colony forming units of wild type SL1344 (L354) were reduced 2 and 4 fold after 30 and 60 minutes, respectively (Figure. 3.1b).

After 30 minutes of exposure to 8 µg/ml of ceftriaxone (MIC) there was a 1-log reduction in the viability of the strains (Figure 3.2a). There was no further significant reduction in viability of the three strains after 60 minutes of exposure to 8 µg/ml of ceftriaxone. Following exposure to 82µg/ml of ceftriaxone (C_{max}), no viable bacteria were retrieved for any strains after 30 or 60 minutes post antibiotic exposure.

There was no effect on the viability of wild type SL1344 (L354), SL1344 *tolC::aph* (L109), and SL1344 ∆*aroA* (L730) strains on exposure to both 0.5 (MIC) and 2 µg/ml (C_{max}) of tetracycline. Statistical analysis showed that after 30 and 60 minutes exposure to antibiotics, the number of viable cells for all three strains was the same as at time zero (Figure 3.3a and b).

As with tetracycline, when incubated with azithromycin at both MIC and C_{max} concentrations (2.0 and 1.43 µg/ml, respectively), there was no inhibition of growth for the three strains of *Salmonella*. Furthermore, there were no differences in the number of colony forming units of both SL1344 *tolC::aph* (L109) and SL1344 Δ*aroA* (L730) after 30 and 60 minutes of exposure (Figure 3. 4a and b).

There were no changes in the viability of (wild type) L354, SL1344 *tolC::aph* (L109) and SL1344 Δ*aroA* (L730) after 30 minutes incubation with 8µg/ml (MIC) streptomycin. However, after 60 minutes exposure, a non-significant decrease in the number of viable bacteria was observed for SL1344 Δ*aroA* (L730). The viability of SL1344 *tolC::aph* (L109) and wild type SL1344 (L354) was not affected at the C_{max} concentration of 30µg/ml. There was no inhibition of growth or reduction in the number of viable bacteria after 30 and 60 minutes of streptomycin exposure (Figure 3.5a and b).

Figure 3.1 The effect of ciprofloxacin at MIC and C$_{max}$ on *Salmonella* in liquid broth.

a.   Growth of *Salmonella* in broth with ciprofloxacin at 0.015 µg/ml (MIC)
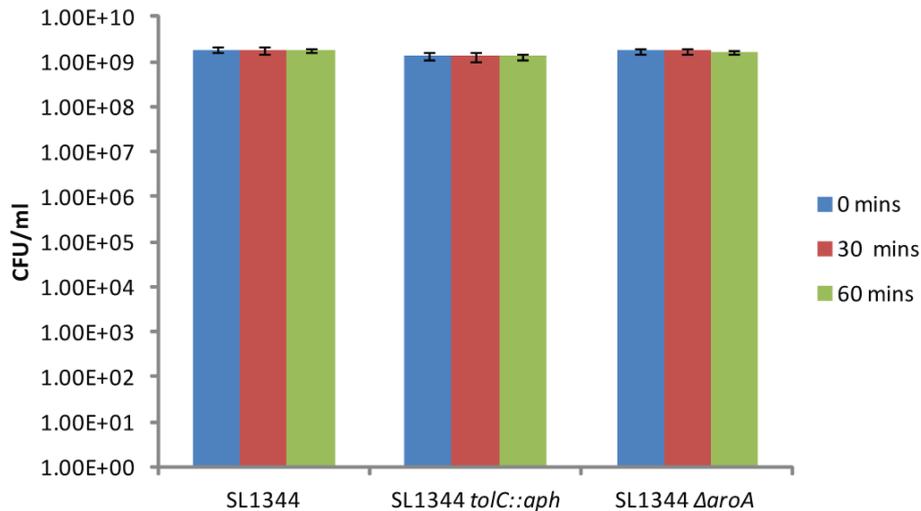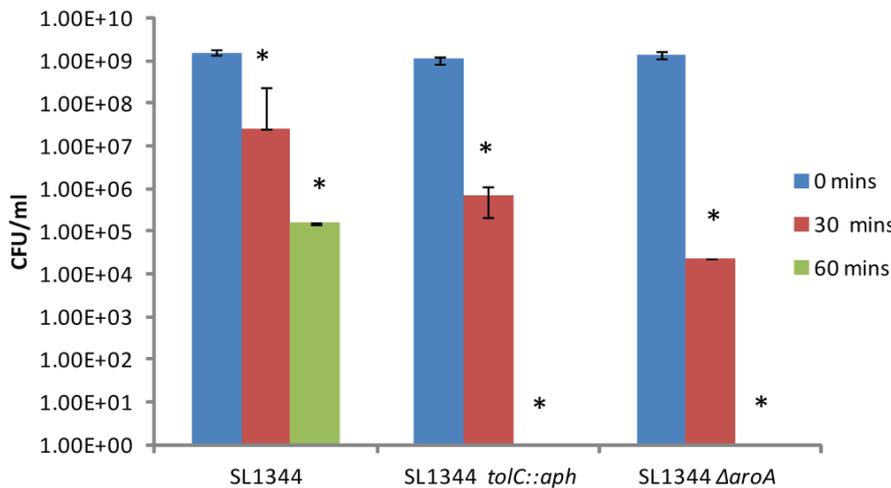
b.   Growth of S*almonella* in broth with ciprofloxacin at 5 µg/ml (C$_{max}$)

The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, red and green bars represent colony forming units at 0, 30 and 60 minutes antibiotic exposure respectively. A Student's t-test was carried out to compare viable counts at the start of the experiments and at 30 and 60 minutes. The * indicates a significant difference (*p*<0.05), all other comparisons were considered non-significant, n=3.

Figure 3.2 The effect of ceftriaxone at MIC and C$_{max}$ on *Salmonella* in liquid broth.

a. Growth of *Salmonella* in broth with ceftriaxone at 8µg/ml (MIC)
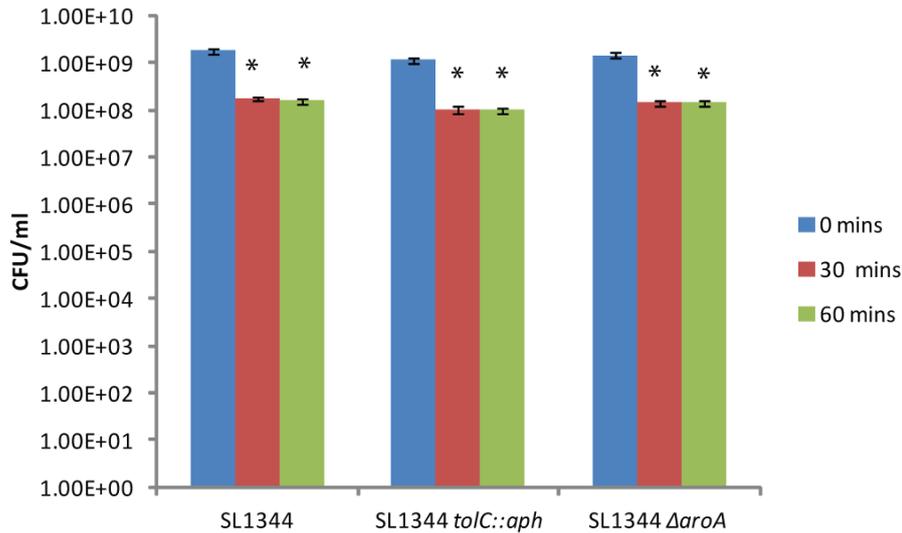
b. Growth of *Salmonella* in broth with ceftriaxone at 82 µg/ml (C$_{max}$)
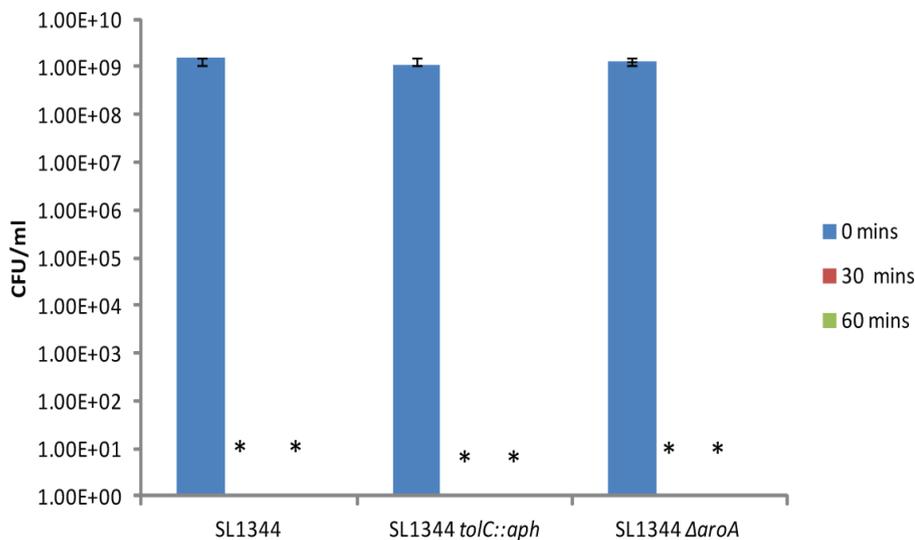
The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, red and green bars represent colony forming units at 0, 30 and 60 minutes antibiotic exposure respectively. A Student's t-test was carried out to compare viable counts at the start of the experiments and at 30 and 60 minutes. The * indicates a significant difference (*p*<0.05), all other comparisons were considered non-significant, n=3.

Figure 3.3 The effect of tetracycline at MIC and C$_{max}$ on *Salmonella* in liquid broth.

a.   Growth of *Salmonella* in broth with tetracycline at 0.05 µg/ml (MIC)
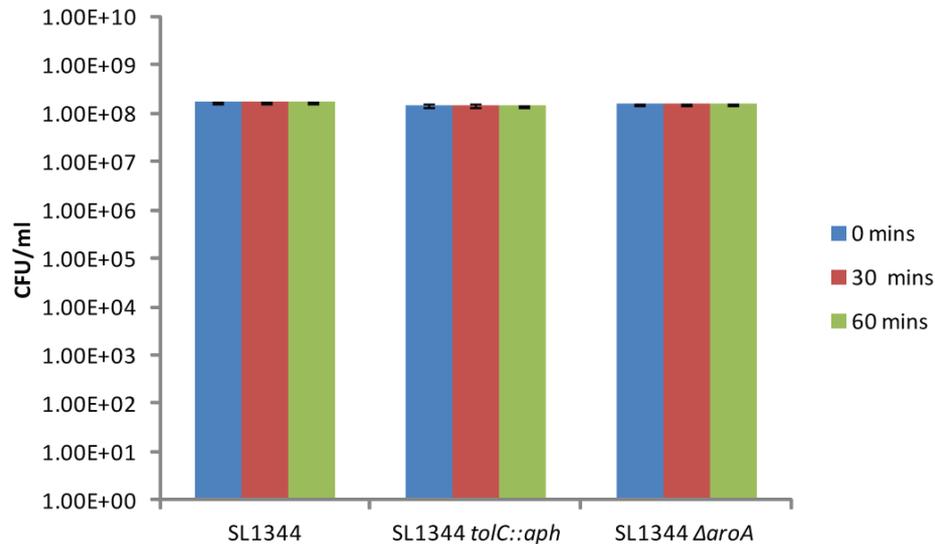
b.   Growth of *Salmonella* in broth containing tetracycline 2.0µg/ml (C$_{max}$)
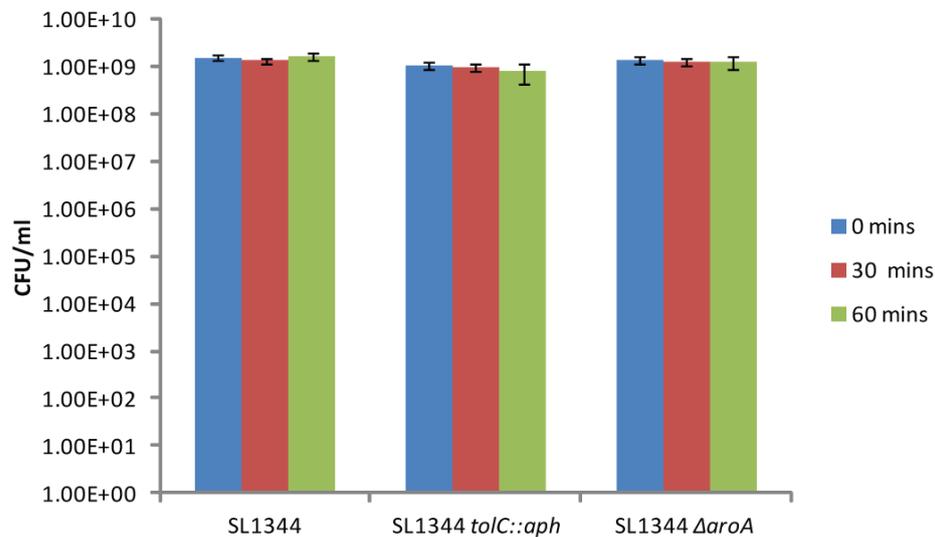
The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, red and green bars represent colony forming units at 0, 30 and 60 minutes antibiotic exposure respectively. A Student's t-test was carried out to compare viable counts at the start of the experiments and at 30 and 60 minutes. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant ($p>0.05$) n=3.

Figure 3.4 The effect of azithromycin at MIC and C$_{max}$ on *Salmonella* in liquid broth.

a.   Growth of *Salmonella* in broth with azithromycin at 2.0 µg/ml (MIC)
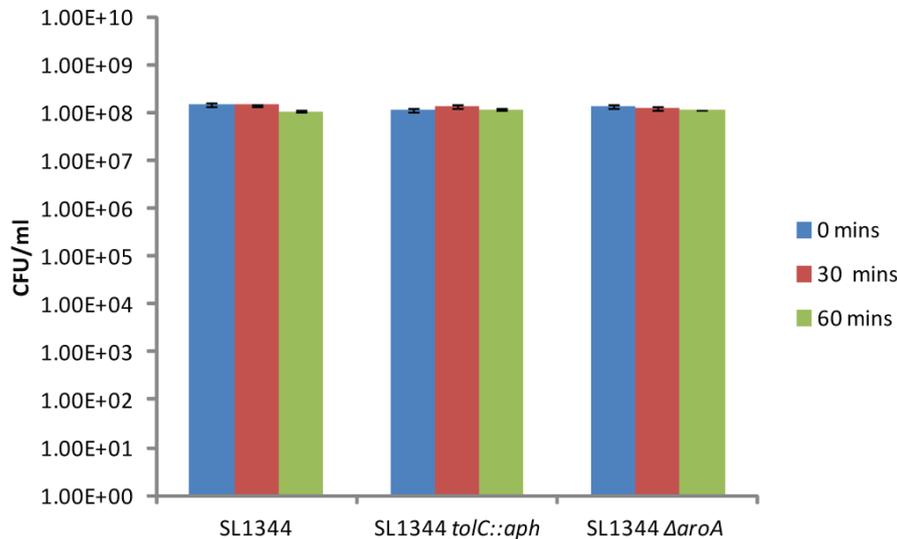
b.   Growth of *Salmonella* in broth with azithromycin at 1.43 µg/ml (C$_{max}$)
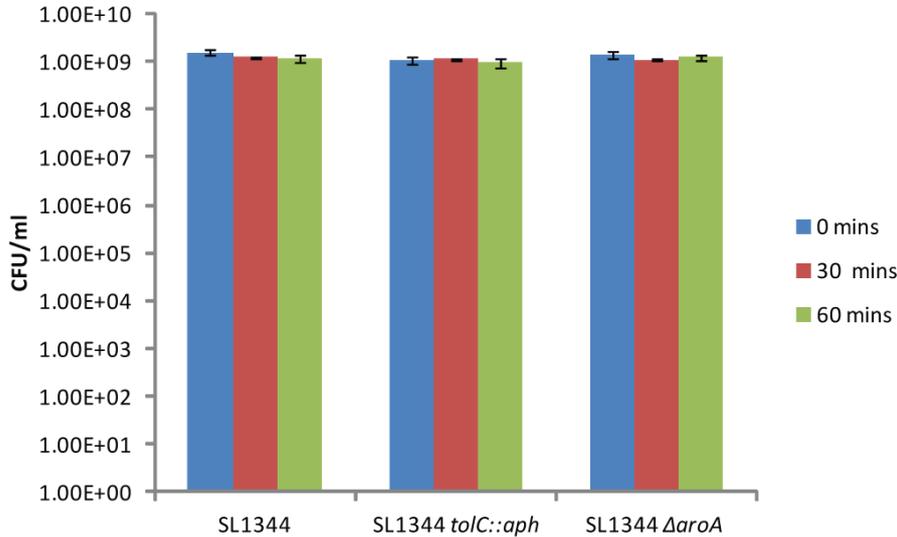
The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, red and green bars represent colony forming units at 0, 30 and 60 minutes antibiotic exposure respectively. A Student's t-test was carried out to compare viable counts at the start of the experiments and at 30 and 60 minutes. The * indicates a significant difference ($p < 0.05$), all other comparisons were considered non-significant, n=3.

Figure 3.5 The effect of streptomycin at MIC and C$_{max}$ on *Salmonella* in liquid broth

a.   Growth of *Salmonella* in broth with streptomycin at 8 µg/ml (MIC)
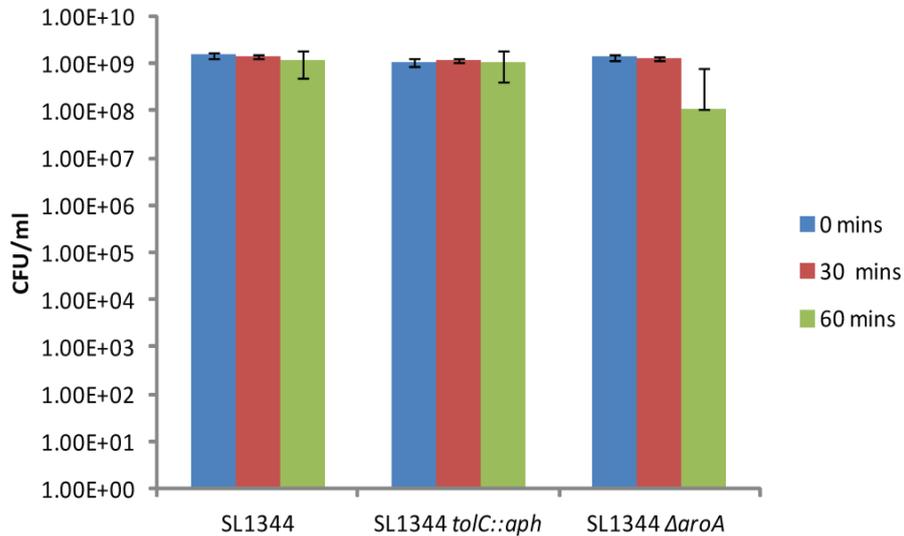
b.   Growth of *Salmonella* in broth with streptomycin at 30 µg/ml (C$_{max}$)
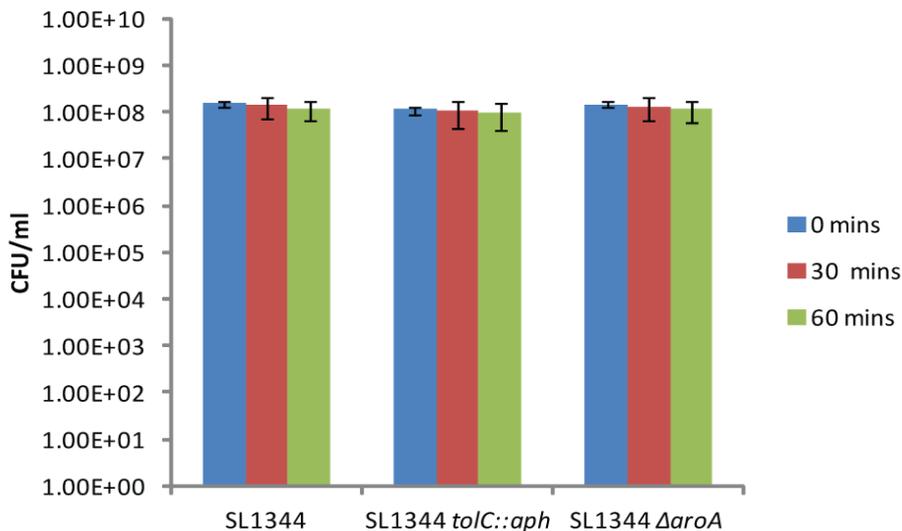
The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, red and green bars represent colony forming units at 0, 30 and 60 minutes antibiotic exposure respectively. A Student's t-test was carried out to compare viable counts at the start of the experiments and at 30 and 60 minutes. The * indicates a significant difference ($p < 0.05$), all other comparisons were considered non-significant, n=3.

Table 3.2 Summary of effects of five antibiotics at MIC and C_{max} concentrations on three strains of *Salmonella* Typhimurium.

Salmonella Typhimurium SL1344

Antibiotic	MIC effect	C_{max} effect
Ciprofloxacin	=	↓
Tetracycline	=	=
Ceftriaxone	↓	↓
Azithromycin	=	=
Streptomycin	=	=

Salmonella Typhimurium SL1344 *tolC::aph* (L109)

Antibiotic	MIC effect	C_{max} effect
Ciprofloxacin	=	↓
Tetracycline	=	=
Ceftriaxone	↓	↓
Azithromycin	=	=
Streptomycin	=	=

Salmonella Typhimurium SL1344 ΔaroA (L730)

Antibiotic	MIC effect	C_{max} effect
Ciprofloxacin	=	↓
Tetracycline	=	=
Ceftriaxone	↓	↓
Azithromycin	=	=
Streptomycin	↓	=

↓ represents decrease in CFU/ml of bacteria compared to t = 0 min.

= represents no change in CFU/ml of bacteria compared to t = 0 min.

3.5   Discussion.

In the experiments described in chapter three, antimicrobial activities of the five antibiotics were tested against bacteria in the absence of immune cells. To establish the direct effect of these antibiotics at this time point, the experiments described in this chapter were carried out under similar conditions as in the neutrophil killing assay (section 5.5). Therefore, in future experiments with peripheral blood neutrophils, any observation would be due to an effect on the immune cells. Within the 2 hour incubation period, ciprofloxacin and ceftriaxone had either cidal or static effects on the numbers of viable *Salmonella* grown in liquid broth, whereas azithromycin, tetracycline and streptomycin had no effects on bacterial viability. This was expected as the short incubation time is insufficient to show the effect of all antibiotics tested, as antimicrobial effects are usually observed over a 16 – 24 hour incubation period. The 2 hour incubation period was used to mimic the T_{max} of the antibiotics *in vivo*. These experiments were done in parallel to those described in chapters 5, section 5.5 but were presented in separate chapters in order to simplify presentation and interpretation of data for the reader.

The experiments described herein were carried out to determine the interaction between antibiotics (at clinically relevant concentrations) and bacteria alone. Apart from antibiotic concentration, temperature was regulated. Usually the average temperature of the human body is 37˚C and as the antibiotics tested are stable at this temperature, the assays were carried out at this temperature.

The infectious dose of *Salmonella* in humans is reported to be between 10^5 and 10^{10} colony forming units (Blaser and Newman, 1982). Georgiade (1983) also reported that infections occur when populations of bacteria reach 10^5 CFU of bacteria units

per gram of tissue (Georgiade, 1983). In line with this, a bacterial concentration of 10^5 CFU was used in these experiments. In MIC experiments, time of exposure to antibiotics is ~18 hours. However, in the human body, the time to reach peak serum concentration (T_{max}) for the antibiotics tested range from 1-2 hours, hence 60 minutes exposure period was used in these assays (Lubasch et al., 2000, Rebuelto et al., 2002, Yang et al., 2007).

The killing of *Salmonella* Typhimurium by five classes of different experiments was assessed by *in vitro* experiments determining the viable counts after antibiotic exposure and in liquid broth to understand the effects of antibiotics on bacteria in the absence of immune cells. The C_{max} concentration of antibiotics was selected because this is the concentration of antibiotics that bacteria are exposed to following administration of the drug in the human host.

Antimicrobial agents such as ciprofloxacin and ceftriaxone are effective against *Salmonella* Typhimurium (Brunner and Zeiler, 1988, Bryan et al., 1985) and are used in the treatment of infections caused by this organism. Other antibiotics such as azithromycin, tetracycline and streptomycin have also been tested for their activity against strains of *Salmonella* (Lugo-Melchor et al., 2010, Butler and Girard, 1993). However, tetracycline is not used clinically to treat infections caused by *Salmonella* Typhimurium due to bacterial resistance to this drug (Brunelle et al., 2013, Kariuki et al., 1993). Although azithromycin is used as an alternative for the treatment of typhoid fever in cases of decreased susceptibility to ciprofloxacin (Vlieghe et al., 2012), it did not show efficacy against *Salmonella in vitro* in the experiments reported herein in after 60 minutes. The MIC of azithromycin for *Salmonella* isolates (*in vitro* after 18 hours is 4 – 16 µg/ml (Sjolund-Karlsson et al., 2011). The lack of detectable

antibacterial effect after two hours exposure is not uncommon for macrolides (Schachter, 1991). This therefore allowed investigation of the effect of this agent upon the response of specific immune cells to *Salmonella* challenge, independent of their antibacterial effects. Moreover, data was obtained under similar conditions as described in chapter 5.5 (albeit in the absence of any immune cell). Growth kinetics curves from assays investigating the effect of azithromycin on *Salmonella*, showed that azithromycin did not inhibit the growth of *Salmonella* at 30 or 60 minutes of incubation (data not shown). Future work should include determining the viable counts of *Salmonella* at longer periods of incubation with azithromycin. There was no decrease in viability of SL1344 exposed to tetracycline and streptomycin as within the time points investigated, bacteria are (Leung and Finlay, 1991) not susceptible to these antibiotics.

Compared to tetracycline, azithromycin and streptomycin, *Salmonella* was killed by ciprofloxacin at 5 µg/ml (C_{max}) only and ceftriaxone at 8 µg/ml (MIC). Ceftriaxone was also bactericidal at 82 µg/ml (C_{max}). This is in agreement with work by Luster et al., (1997) where they showed that ceftriaxone exhibited bactericidal activity in a time and concentration dependent manner, though against *Streptococcus pneumonia* (Lutsar et al., 1997). Similarly, when Tang et al., (2011) compared killing kinetics of tigecycline (belonging to the glycylcycline class of antibiotics; a derivative of the tetracyclines), ceftriaxone and ciprofloxacin against two *Salmonella* isolates S129-42 and S129-25, they showed that ceftriaxone (8 µg/ml) and ciprofloxacin (1 µg/ml) had greater bactericidal activity than tigecycline (2 µg/ml).

Traditionally, ciprofloxacin and ceftriaxone are recommended for treating *Salmonella* infections. Not surprisingly, of the five antibiotics tested, ciprofloxacin and ceftriaxone

killed more bacteria. Overall, the data show that at the C_{max} concentration of ciprofloxacin and ceftriaxone inhibited the growth of the strains although after 60 minutes exposure they did not completely eradicate all *Salmonella*.

The limitations of these experiments carried out in liquid broth include that *in vitro* experiments do not exactly replicate what takes place in a host as they do not account for;

a. Growth factors, proteases, antimicrobial peptides.

b. Fluctuations of the drug concentration at different sites in the host.

c. Increases in body temperature during an infection.

Bacteria also behave differently *in vivo* and *in vitro*, for instance due to altered bacterial metabolism and growth rate. This may account for the differences in the efficacy of antibiotics under both conditions (de Araujo et al., 2011).

3.6   Key findings

1. Ciprofloxacin was more effective at killing bacteria at the C_{max} concentration than at the MIC.

2. Ceftriaxone was the most bactericidal agent tested (Table 3.1).

3. Tetracycline, azithromycin and streptomycin did not alter the viability of the bacterial strains.

CHAPTER FOUR

4 The adhesion to and invasion of *Salmonella* Typhimurium in antibiotic pre-treated murine macrophages.

4.1 Background.

Within the host, the innate immune system provides the first line of defence against invading *Salmonella*. Cells of the innate immune system such as neutrophils and macrophages cooperate in the rapid recognition and elimination of invading *Salmonella* through processes such as phagocytosis-mediated killing and induction of inflammation (Broz et al., 2012).

Macrophages are important in *Salmonella* pathogenicity as adhesion to and invasion of these cells and adaption of these bacteria to life within the host cells are key steps in the dissemination and progress of infection (Shi and Casanova, 2006). As was shown from data described in Chapter 3, antibiotics at concentrations corresponding to the peak serum concentration (C_{max}) in the host did not completely inhibit the growth of *Salmonella* after 60 minutes of exposure in liquid broth. Therefore, *in vitro* infection assays of macrophages were carried out to investigate whether the same antibiotic concentrations influence the interaction between *Salmonella* and these cells.

4.2 Hypothesis.

Adhesion to and invasion of *Salmonella* to macrophages is influenced by the presence of antibiotics.

4.3   Aim.

To investigate if the ability of *Salmonella* to adhere to and invade cell line (J774) and murine primary macrophages (bone marrow derived macrophages) is altered by exposure of macrophages to antibiotics prior to infection with bacteria.

4.4   Adhesion to and invasion of *Salmonella* in antibiotic treated J774 mouse macrophages.

The data described herein are based on infection assays, which have been used widely to study the adhesion and invasion of host cells by bacterial pathogens (Edwards and Massey, 2011, Dibb-Fuller et al., 1999). In these experiments, bacterial adhesion is a measure of the number of bacteria that have attached to host cell surfaces while invasion measures the number of bacteria that have entered the cells.

The abilities of wild type SL1344 (L354), SL1344 *tolC::aph*, (L109), SL1344 Δ*aroA*, (L730) and SL1344 SPI-1::*aph* (L1449) to adhere to, and invade, J774 macrophages after exposure to ciprofloxacin, tetracycline, ceftriaxone, azithromycin and streptomycin was investigated. As in Chapter 3, the strains in which the *tolC* and *aroA* genes were inactivated or deleted were chosen as controls because they have been previously shown to be less virulent and less invasive than wild type SL1344, making them suitable for use in these tissue culture infection assays (Section 3.4). The fourth strain, SL1344 SPI-1::*aph* (L1449) was included as it has been previously shown that SPI-1 mutants have impaired ability to invade host cells (Pavlova et al., 2011). Using these strains allows discrimination between invasion levels of *Salmonella* in the presence or absence of antibiotics.

Data from experiments measuring adhesion and invasion of *Salmonella* into J774 macrophages in this chapter were analysed using Graphpad Prism software, and confirmed to be normally distributed.

4.4.1 Ciprofloxacin

When macrophages were pre-treated with 0.015 µg/ml of ciprofloxacin (MIC), there was a significant increase in the CFU/ml of wild type SL1344 and SPI-1::*aph* (L1449) that adhered to the macrophages (Figure 4.1a). However, adhesion was not altered for either SL1344 *tolC::aph* (L109) or SL1344 Δ*aroA* (L730) (Figure 4.1a). In the absence of antibiotic pre-exposure, there was no difference in the number of SL1344 or SL1344 SPI-1*::aph* that invaded J774 macrophages. However, consistent with published data, SL1344 *tolC::aph* invaded poorly (Blair et al., 2009). Compared with no antibiotic treatment, greater numbers of SL1344 *tolC::aph* invaded ciprofloxacin treated macrophages than untreated macrophages. The numbers of SL1344 Δ*aroA* that invaded the ciprofloxacin treated macrophages was significantly lower than non-antibiotic treated macrophages (Figure 4.1b), while invasion of SL1344 SPI-1*::aph* was not altered in both ciprofloxacin treated or untreated macrophages.

When J774 macrophages were pre-treated with 5 µg/ml of ciprofloxacin (C_{max}), compared to untreated macrophages there was a significant increase in the number of SL1344 that adhered to macrophages. However, there was a decrease in adhesion of SL1344 SPI-1:aph to ciprofloxacin treated macrophages. There was no difference in the numbers of SL1344 *tolC::aph* and SL1344 Δ*aroA* that adhered to ciprofloxacin treated macrophages (Figure 4.2a). The numbers of SL1344, SL1344 Δ*aroA* and SL1344 SPI-1*::aph* that invaded the 5 µg/ml ciprofloxacin pre-treated macrophages was significantly lower than untreated macrophages (Figure 4.2b).

However, greater numbers of SL1344 *tolC::aph* invaded macrophages pre-exposed to 5 µg/ml of ciprofloxacin than in non-ciprofloxacin exposed macrophages.

Figure 4.1 Effect of 0.015 µg/ml ciprofloxacin (MIC for SL1344) on adhesion to and invasion of wild type *S*. Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A

B

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded ciprofloxacin treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant ($p>0.05$), n>3.

Figure 4.2 Effect of 5 μg/ml ciprofloxacin (C_{max}) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A

B

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded ciprofloxacin treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

4.4.2 Ceftriaxone

Compared with no antibiotic treatment, significantly higher numbers of SL1344 and SL1344 Δ*aroA* adhered to macrophages treated with 8 µg/ml of ceftriaxone (Figure 4.3a). However, adhesion of SL1344 *tolC::aph* was reduced, while adhesion of SL1344 SPI-1::*aph* was not altered in macrophages pre-treated with the same concentration of ceftriaxone. The numbers of SL1344 *tolC::aph* that invaded ceftriaxone treated macrophages was significantly greater than untreated macrophages. However, invasion of SL1344 and SL1344 Δ*aroA* was decreased in macrophages pre-treated with 8µg/ml of ceftriaxone, while adhesion of SL1344 SPI-1::*aph* was not altered in both treated and untreated macrophages (Figure 4.3b).

In J774 macrophages pre-treated with 82 µg/ml of ceftriaxone (C_{max}), there was a significant increase in the CFU/ml of wild type SL1344 and SL1344 Δ*aroA* that adhered to macrophages than in non-antibiotic treated macrophages. Adhesion of SPI-1::*aph* was decreased while adhesion of SL1344 *tolC::aph* was not changed in the ceftriaxone treated or untreated macrophages (Figure 4.4a). However, invasion of SL1344, SL1344 Δ*aroA* and SL1344 SPI-1*::aph* to J774 macrophages was not altered in ceftriaxone pre-treated (82 µg/ml) or untreated macrophages (Figure 4.4b). Greater numbers of SL1344 *tolC::aph* invaded macrophages treated with 82 µg/ml of ceftriaxone than in untreated macrophages.

Figure 4.3 Effect of 8 μg/ml ceftriaxone (MIC for SL1344) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A

B

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded ceftriaxone treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference (p<0.05), all other comparisons were considered non-significant, n>3.

Figure 4.4 Effect of 82 µg/ml ceftriaxone (C$_{max}$) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 tolC::aph (L109), SL1344 ∆*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.
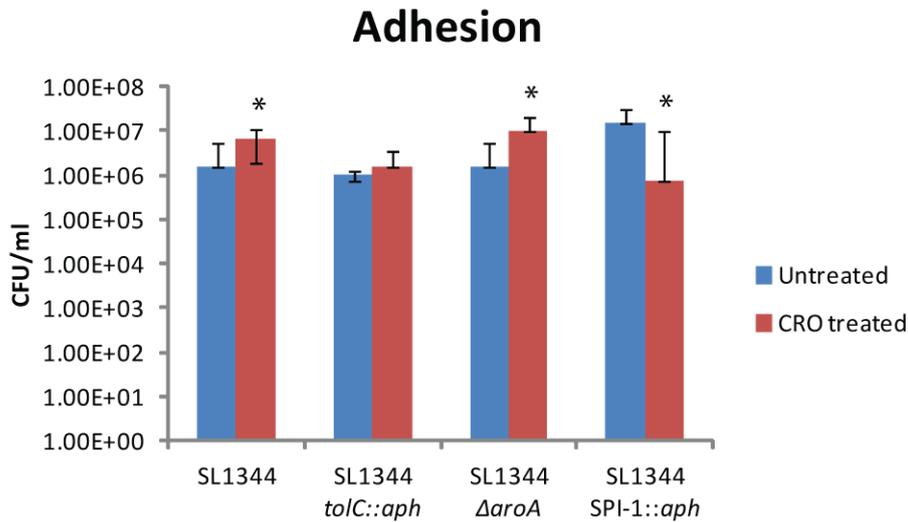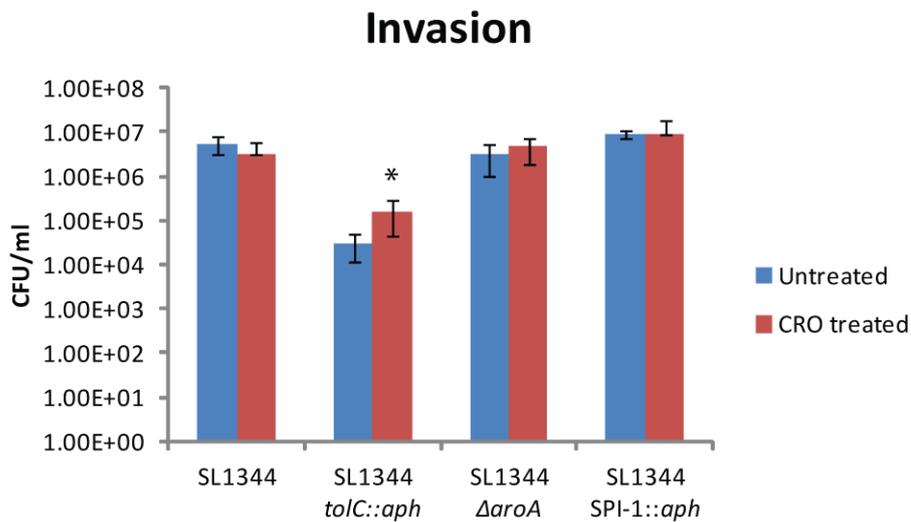
A.

B.

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded ceftriaxone treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference (p<0.05), all other comparisons were considered non-significant, n>3.

4.4.3 Tetracycline

When macrophages were pre-treated with 0.5 µg/ml of tetracycline (MIC), there was a significant increase in adhesion of all strains, except SL1344 (Figure 4.5a). There was an increase in the number of SL1344 *tolC::aph* that invaded tetracycline treated macrophages than untreated macrophages. However, there were no changes between the numbers of bacteria that invaded tetracycline treated and untreated macrophages for SL1344, SL1344 Δ*aroA* and SL1344 SPI-1*::aph* (Figure 4.5 b).

There was no significant difference between the numbers of SL1344, SL1344 Δ*aroA* or SL1344 SPI-1::*aph* that adhered to either tetracycline (2 µg/ml) pre-treated macrophages or untreated macrophages (Figure 4.6a). However, compared with untreated macrophages, lower numbers of SL1344 *tolC::aph* adhered to macrophages treated with 2 µg/ml of tetracycline (C_{max}). The numbers of SL1344 *tolC::aph* that invaded macrophages treated with tetracycline was significantly higher than in untreated macrophages. The invasion numbers of SL1344, SL1344 Δ*aroA* and SL1344 SPI-1*::aph* was not altered in tetracycline treated (2 µg/ml) compared with untreated macrophages (Figure 4.6b).

Figure 4.5 Effect of 0.5 µg/ml tetracycline (MIC for SL1344) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A

B

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded tetracycline treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

Figure 4.6 Effect of 2 µg/ml tetracycline (C$_{max}$) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A.

B.

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded tetracycline treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

4.4.4 Azithromycin

Adhesion of all four strains to macrophages pre-treated with 2 µg/ml of azithromycin was significantly lower than in untreated macrophages (Figure 4.7a). When macrophages were treated with azithromycin, there was a significant decrease in the CFU/ml of SL1344 and SL1344 Δ*aroA* that invaded these macrophages. However, there was no significant difference between the CFU/ml of SL1344 *tolC::aph* and SL1344 *SPI-1::aph* that invaded azithromycin pre-treated or untreated macrophages (Figure 4.7b).

When macrophages were pre-treated with 1.4 µg/ml of azithromycin, greater numbers of SL1344 adhered than in untreated macrophages. There was no difference in the numbers of SL1344 *tolC::aph*, SL1344 Δ*aroA* and SL1344 SPI-*1::aph* that adhered to azithromycin treated and untreated macrophages (Figure 4.8a). There was significant decrease in the numbers of SL1344, SL1344 Δ*aroA* and SL1344 SPI-1*::aph* that invaded azithromycin treated macrophages but not in SL1344 *tolC::aph* (Figure 4.8b)*.*

Figure 4.7 Effect of 2 μg/ml azithromycin (MIC for SL1344) on adhesion to and invasion of wild type S. Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A.

B.
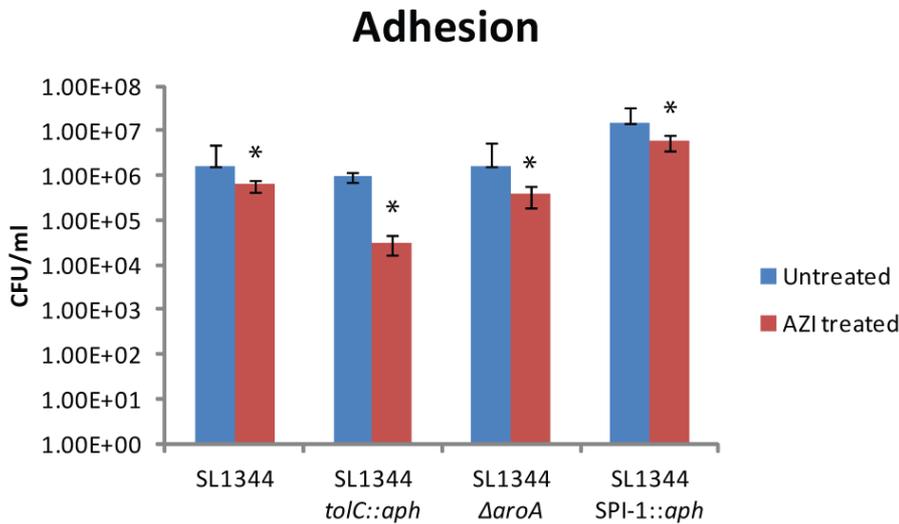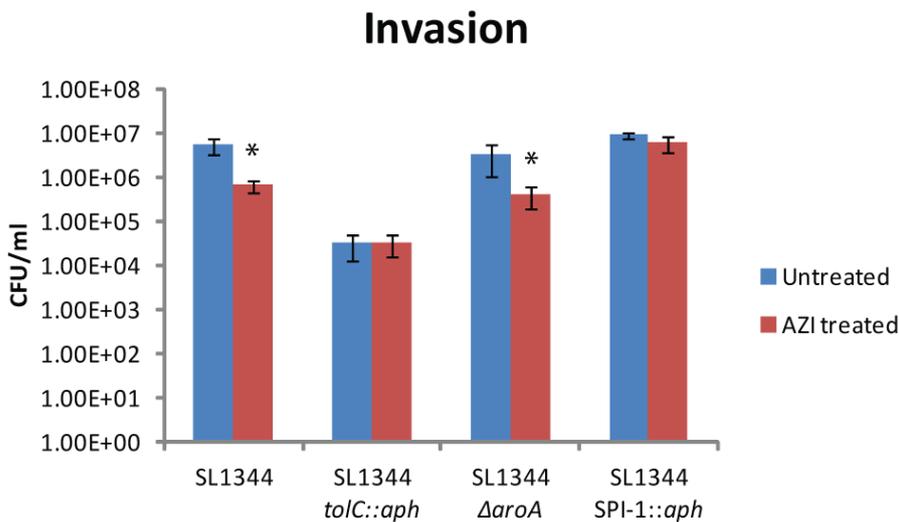
The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded azithromycin treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

Figure 4.8 Effect of 1.4 μg/ml azithromycin (C$_{max}$) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine J774 macrophages.

A.

B.

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded ceftriaxone treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

4.4.5 Streptomycin

Exposure of macrophages to 8 μg/ml of streptomycin did not alter the adhesion of SL1344, SL1344 *ΔaroA* and SL1344 SPI-1::*aph*.   However, adhesion of SL1344 *tolC::aph* was significantly lower in streptomycin treated macrophages than untreated macrophages (Figure 4.9a). The numbers of SL1344 and SL1344 SPI-1::*aph* that adhered to J774 macrophages was not altered when the macrophages were pre-treated with 8 μg/ml of streptomycin. Further, the invasion of SL1344 *tolC::aph* and SL1344 SPI-1::*aph* was significantly greater in macrophages pre-treated with 8 μg/ml of streptomycin (Figure 4.9b).

In macrophages treated with 30μg/ml of streptomycin (C_{max}), there was significantly increased adhesion of the wild type SL1344, SL1344 *ΔaroA* and SL1344 SPI-1*::aph* strains. Adhesion of SL1344 *tolC::aph* was not affected by treatment of macrophages with 30 μg/ml of streptomycin (Figure 4.10a). Invasion of SL1344 *tolC::aph* was significantly higher in streptomycin treated macrophages than in non-treated macrophages. Streptomycin treatment did not alter invasion numbers of SL1344, SL1344 *ΔaroA* and SL1344 SPI-1::*aph* (Figure 4.10b).

Figure 4.9 Effect of 8 µg/ml streptomycin (MIC for SL1344) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine cell line J774 macrophages.

A.

B.

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded streptomycin treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

Figure 4.10 Effect of 30 µg/ml streptomycin (C$_{max}$) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354), SL1344 *tolC::aph* (L109), SL1344 Δ*aroA* (L730) and SL1344 SPI-1*::aph* (L1449) in murine cell line J774 macrophages.

A.

B.

The data presented in the charts are means of at least three individual experiments performed in triplicate (+/- SD). The blue bars represent colony forming units of bacteria that adhered to or invaded untreated macrophages while red bars represent bacteria that adhered to or invaded streptomycin treated macrophages. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n>3.

4.5   Adhesion to and invasion of *Salmonella* in antibiotic treated bone marrow derived macrophages.

The experiments described in section 4.4, comparing adhesion and invasion of four *Salmonella* strains to antibiotic treated macrophages, typically showed increased bacterial adhesion to antibiotic treated macrophages than untreated counterparts. Ciprofloxacin and cefriaxone were the two antibiotics that most frequently affected bacterial adhesion to antibiotic treated J774 macrophages. Hence, these two antibiotics were further investigated for their effect on adhesion and invasion of *Salmonella* to mouse bone marrow derived macrophages (BMDMs). Although J774 macrophage cell line is a well-established model system in cell biology and immunology, they are cell lines that have been immortalised and maintained by genetic manipulation. Hence to validate the findings from infection assays with these cells (Section 4.4), mouse bone marrow derived macrophages (BMDMs) were isolated and used in the same tissue culture infection assays as the J774 macrophages cells of murine origin, and allowed assessment of the effects of the antibiotics reported in using murine J774 cells in tissue culture. The BMDMs were treated with ciprofloxacin and ceftriaxone at C_{max} concentrations i.e. 5 and 82 µg/ml respectively. Only the wild type SL1344 was used to infect the BMDMs because with this strain, there was consistent increased adhesion to J774 macrophages pre-exposed to ciprofloxacin and ceftriaxone at both concentrations (Table 4.1).

Treatment of macrophages with ciprofloxacin and ceftriaxone significantly increased bacterial adhesion to these cells (Figure 4.11a). Conversely, there was a significant decrease in the invasion of SL1344 to ciprofloxacin and ceftriaxone treated macrophages than in untreated controls (Figure 4.11).

129

Figure 4.11 Effect of 5 µg/ml of ciprofloxacin and 82 µg/ml ceftriaxone (C$_{max}$) on adhesion to and invasion of wild type *S.* Typhimurium SL1344 (L354) in bone marrow derived mouse macrophages.

A
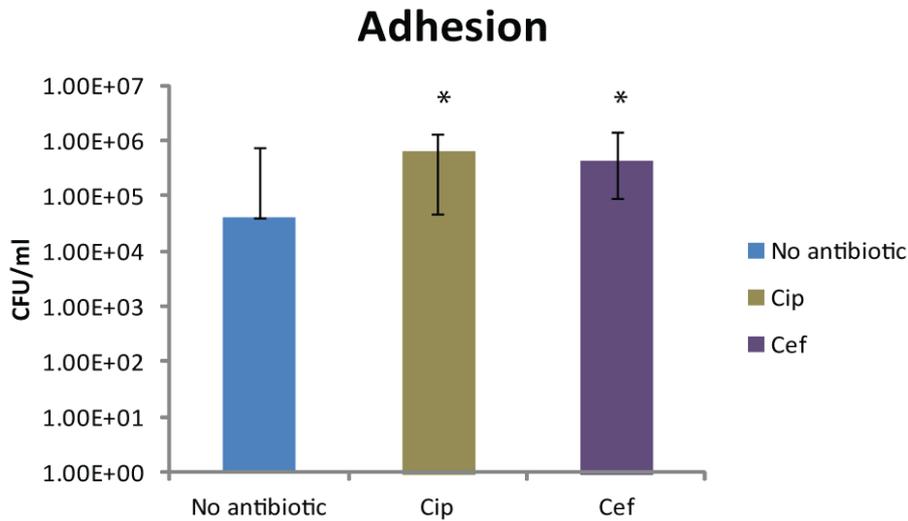

B
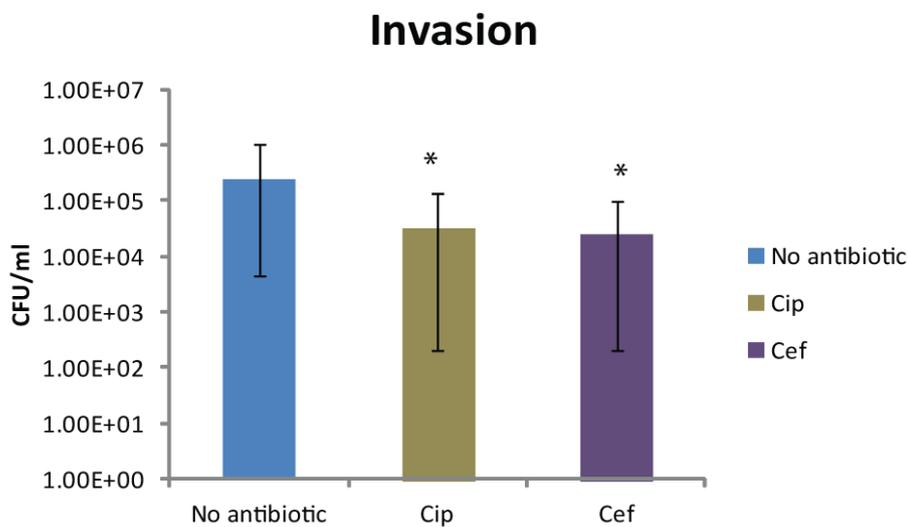
The data presented in the charts are means of three individual experiments performed in triplicate (+/- SD). The blue, green and purple bars represent colony forming units of bacteria that adhered to or invaded untreated, ciprofloxacin and ceftriaxone treated macrophages respectively. A Student's t-test was carried out to compare viable counts for treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Compared to wild type parent strain, there were no significant differences in the adherence of the strains used to distinguish between adherence and invasion by the bacteria versus phagocytosis by the macrophages (SL1344 *tolC::aph* and SL1344 Δ*aroA*) to non-antibiotic treated macrophages. However, the SPI-1 mutant strain adhered in significantly higher numbers than all other strains (Figures 4.1a - 4.10b, blue bars).

There was a consistent increase in the adhesion of wild type pathogenic SL1344 to antibiotic pre-treated macrophages with both concentrations of ciprofloxacin and ceftriaxone. Adhesion of this strain was also increased when macrophages were pre-treated with C_{max} concentrations of azithromycin and streptomycin. However, lower numbers of SL1344, adhered to macrophages treated with MIC of azithromycin. There were no changes in the number of adhered bacteria for macrophages treated with tetracycline and at the MIC of streptomycin (Table 4.1).

Consistent with published work by Blair et al, (2009), SL1344 *tolC::aph* had significantly deceased ability to invade non-antibiotic treated J774 macrophages compared with the wild type parent strain, SL1344 (Figures 4.1a - 4.10b, blue bars), while adhesion was not altered. However, except azithromycin, when macrophages were pre-exposed to antibiotics, there were significant increases in the numbers of SL1344 *tolC::aph* that invaded the cells compared with untreated macrophages (Table 4.1).

Unlike the SL1344 *tolC::aph* mutant, the abilities of SL1344, SL1344 Δ*aroA* or SL1344 SPI-1::*aph* to adhere to, or invade, antibiotic pre-exposed macrophages varied between the antibiotics at MIC and C_{max}. Notably, for the wild type SL1344 there was increased adhesion when macrophages were pre-exposed to C_{max} and

MIC concentrations of ciprofloxacin (0.015 and 2 μg/ml, respectively) and ceftriaxone (8 and 82 μg/ml respectively). Similarly, more SL1344 SPI-1::*aph* adhered to macrophages pre-exposed to C_{max} and MIC of ciprofloxacin, while more SL1344

Table 4.1 Summary table of data for adhesion to and invasion of *Salmonella* in antibiotic treated J774 macrophages.

Antibiotic pre-treatment	Adhesion				Invasion			
	SL1344	SL1344 *tolC::aph*	SL1344 *ΔaroA*	SL1344 SPI-1::*aph*	SL1344	SL1344 *tolC::aph*	SL1344 *ΔaroA*	SL1344 SPI-1::*aph*
Ciprofloxacin 0.015 µg/ml	↑	=	=	↑	=	↑	↑	=
Ciprofloxacin 5 µg/ml	↑	=	=	↑	↓	↑	↓	↓
Ceftriaxone 8 µg/ml	↑	↓	↑	=	↓	↑	↓	=
Ceftriaxone 82 µg/ml	↑	=	↑	↓	=	↑	=	=
Tetracycline 0.5 µg/ml	=	↑	↑	↑	=	↑	=	=
Tetracycline 2 µg/ml	=	↑	=	=	=	↑	=	=
Azithromycin 2 µg/ml	↓	↓	↓	↓	↓	=	↓	=
Azithromycin 1.4 µg/ml	↑	=	=	=	↓	=	↓	↓
Streptomycin 8 µg/ml	=	↓	=	=	=	↑	↑	=
Streptomycin 30 µg/ml	↑	=	↑	↑	=	↑	↓	=

↑ represents significant ($p<0.05$) increase in CFU/ml of bacteria compared to no antibiotic treatment.

↓ represents significant ($p<0.05$) decrease in CFU/ml of bacteria compared to no antibiotic treatment.

= represents no change in CFU/ml of bacteria compared to no antibiotic treatment.

4.6   Discussion

My experiments have shown that pre-treatment with antibiotics influence the interaction between immune cells such as macrophages and pathogens e.g. *Salmonella.* Antibacterial agents influence phagocyte-pathogen interactions in two major ways; (i) by interfering directly with phagocytes and (ii) by acting on the pathogen in a manner that makes them more immunogenic or prone to activities of phagocytes (van den Broek, 1989). Pathogens that survive within phagocytes are killed by antibacterial agents that can penetrate these cells, but are difficult to kill by agents that do not penetrate (Mandell and Coleman, 2001). It was suggested by Gemmell, (1993) that β-lactam antibiotics would have no effect on host defences, tetracyclines would dampen the immune response, and fluoroquinolones and certain macrolides would display synergy with the host immune system (Gemmell, 1993).

For these infection assays, wild type SL1344 and three isogenic strains containing mutations in the *tolC*, *aroA* and SPI-1 genes were used.   The *tolC* mutant was constructed in a previous study by disrupting the *tolC* gene through insertion of a kanamycin resistance cassette (Buckley et al., 2006). This strain was used as a control in the infection assays because it is less invasive and less virulent compared to the wild type SL1344, as it has previously been established that *Salmonella* containing mutations in *tolC* have decreased ability to adhere to and invade macrophages (Baucheron et al., 2005, Buckley et al., 2006). The *tolC* strain used in my assays served as a good control for the experiments as has a reduced ability to adhere to, and invade tissue culture cells. Hence, any significant increases in the properties can be attributed to pre-exposure of the macrophages to antibiotics.

aroA is involved in the biosynthesis of chorismic acid. This protein is a common precursor for the biosynthesis of multiple important metabolites such as aromatic amino acids (example phenylalanine, tyrosine, tryptophan, benzoid and naphtoid coenzymes, folate cofactors and siderospores (Dosselaere and Vanderleyden, 2001). *aroA* mutants are less virulent in animal infections models, hence its use as a vaccine strain (Shata et al., 2000). Although growth of *aroA* mutants in intracellular compartments may be affected by the limitation of exogenous aromatic metabolites, this mutation does not affect the ability of bacteria to adhere or invade cultured cells (Chatfield et al., 1992, Lowe et al., 1999). Hence in using this strain that does not have any impairment in adhesion and invasion of host cells as a control, changes in the levels of adhesion or invasion can be attributed to antibiotic effect on the macrophages.

The SPI-1 locus is a 40 kb stretch of DNA located on the *Salmonella* chromosome; it contains genes that encode the type three secretion system (TTSS) responsible for the translocation of virulence proteins from *Salmonella* into the host. These proteins reorganises the host cell and triggers invasion of bacteria into the host cell (Dieye et al., 2009). SPI-1 promotes invasion of *Salmonella* into host cells especially non-phagocytic cells, such as epithelial cells, and also drives the initiation of the inflammatory responses (Dieye et al., 2009).

It has been reported that ciprofloxacin enhances the activities of monocytes and neutrophils by for instance potentiating the killing of microorganism by neutrophils and modulating of cytokines (Bounds et al., 2000, Cacchillo and Walters, 2002, Bamberger et al., 1991). The efficacy of quinolone drugs to penetrate and accumulate within host cells is beneficial in the treatment of infections caused by

intracellular bacteria (Easmon and Crane, 1985). Fluoroquinolones such as ciprofloxacin are widely used due to their broad spectrum of activity for Gram-negative (including *Salmonella*) and Gram-positive bacteria, and their ability to permeate tissues and cells such as macrophages and neutrophils. It has been routinely observed that neutrophils and mononuclear phagocytes take up fluoroquinolones so effectively that the intracellular concentration of the drug sometimes exceeds the extracellular plasma concentration (Easmon et al., 1986, Garraffo et al., 1991).

Ciprofloxacin penetrates immune cells through both simple diffusion or uptake via activation of protein kinase C (Briones et al., 2008). Nonetheless, it is also important to note that cellular accumulation of the antibiotic is not always predictive of the intracellular antimicrobial activity and penetration of phagocytes by antibiotics does not guarantee effective therapeutic activity against the intracellular pathogen. Despite reaching intracellular concentrations (which are often much higher than extracellular concentrations) contrasting views may arise when considering the effect of antibiotics on the immune response to invading pathogen. Van Bambeke et al., (2006) have attributed these divergent views to factors such as impairment of antimicrobial activity when the fluoroquinolone gets to various sub-cellular locations and changes in responsiveness of bacteria to antimicrobial agents within the cells.

I have shown that ceftriaxone enhanced bacterial adhesion of wild type, *aroA* mutant and *tolC* mutant *S*. Typhimurium to pre-treated macrophages but not the SPI-1 mutant. Ceftriaxone is a third generation cephalosporin (a class of beta-lactam antibiotics) used to treat *Salmonella* infections (Frenck et al., 2000). It is also used in the treatment of otitis media (Gauthier et al., 2009) and in combination with

136

macrolides such as azithromycin for the treatment of acute pneumonia (Tamm et al., 2007). Beta-lactams lack the ability to accumulate in phagocytic cells (Prokesch and Hand, 1982, Forsgren and Bellahsene, 1985, Jacobs et al., 1986). According to Carryn et al., (2002) even if beta-lactams can pass through the membranes, they are prevented from accumulating in cells because the cell cytosol is more acidic than the extracellular milieu and may be destroyed. Extrusion of beta-lactams through eukaryotic efflux pumps can cause lack of accumulation within cells and so their activity against intracellular bacteria may be compromised (Carryn et al., 2002).

Macrolides are a class of antibiotics that possess a characteristic macrocyclic lactone ring. By extensive chemical modifications of the natural compound erythromycin A, other semisynthetic derivatives with increased antibacterial spectra and bioavailability have been developed (Bright et al., 1988). Macrolides possess interesting beneficial features, which include accumulation in cells and tissues at high concentrations. Penetration of macrolides into phagocytic cells is by simple diffusion (Briones et al., 2008), or by active uptake mechanism which is mediated by transporter proteins (Parnham et al., 2014). According to Bosnar et al., 2005, this accumulation is beneficial in the treatment of intracellular pathogens and also in the delivery of the drug to sites of infection (Bosnar et al., 2005). Macrolides are also used in short term treatment to enhance phagocytic activities, but their long term use leads to immunosuppression (Minic et al., 2009). Uptake of azithromycin into phagocytic cells is rapid and unsaturable, and it is retained in high amounts in pre-loaded cells. In experiments using two phagocytic cell lines; RAW 246.7 and THP-1 cell lines, it was shown that when extracellular azithromycin was discontinued, the drug was not extruded out of the cell. Three hours post incubation in azithromycin (10 µg/ml), 75%

of the drug still remained cell associated and active (Briones et al., 2008). The data in Figure 4.7a show significant decreases in bacterial adhesion to macrophages pre-exposed to 2 µg/ml of azithromycin, while there was increased adhesion of wild type SL1344 only in macrophages pre-exposed to 1.4 µg/ml of azithromycin. The numbers of wild type, *aroA* mutant and SPI-1 mutant *Salmonella* that invaded macrophages exposed to 1.4 µg/ml of azithromycin was significantly lower than in untreated macrophages. These decreases could be attributed to antimicrobial activities of the cell associated azithromycin. The results for wild-type SL1344 antibiotic treatment indicate increased adhesion and decreased or no effect on invasion. As such, the potential for phagocytosis and destruction of bacteria via phagocytosis would be increased by antibiotic exposure of immune cells.

It is hypothesized that because of their polar aminoglycosides do not penetrate eukaryotic cells nature (Carryn et al., 2002). However, in contradiction, streptomycin is used to treat infections due to intracellular pathogens such as tuberculosis, brucellosis and tularaemia (Maurin and Raoult, 2001). Aminoglycosides are taken up by cells into intracellular compartments through pinocytosis, a process by which cells take up small particles by invagination of the cell membrane, leading to formation of small vesicles within the cell. (Donowitz, 1994). My data showed that streptomycin at 8 µg/ml did not affect the adhesion of any of the strains to macrophages; however invasion of SL1344 *tolC::aph* and SL1344 SPI-1::*aph* to streptomycin treated macrophages was significantly increased compared to control macrophages.

When *in vitro* infection assays were performed using primary macrophages (BMDMs) instead of J774 macrophage cell line, wild type SL1344 adhered to and invaded antibiotic treated primary macrophages (BMDMs) in a similar pattern as seen with the

J774 macrophages. Adhesion of SL1344 to ciprofloxacin and ceftriaxone treated macrophages were significantly increased compared to untreated macrophages, while invasion decreased in antibiotic treated macrophages than in untreated controls. This illustrates that *Salmonella* interacts in a similar way in both cell line and primary immune cells. The demerits of using primary cells in infection assays are that primary cells, for instance neutrophils, have a short life span and proliferate quite slowly compared to macrophage cell lines, which can be cultured for weeks. Further, primary cells derived from different individuals behave differently depending on age, genetics, and tissue of origin or the presence of an underlying disease.

There are a number of caveats to the interpretation of my data by measuring adhesion and invasion. It cannot be easily distinguished if the differences in invasion for instance, were due to impaired ability of the strains to invade efficiently, a defect in intracellular survival in macrophages, or antibacterial effect of residual antibiotics within the macrophages. However, this can be countered by estimating the number of invaded bacteria over a time course, with and without antibiotics to determine bacterial numbers at different points in the experiments. Other factors such as phagocytosis by the macrophages could also influence the invasion results, giving amplified invasion results for strains that have impaired invasive abilities. This could be countered by exposing the macrophages to cytochalasin B a known phagocytosis inhibitor (Finlay et al., 1991).

Further, I investigated whether the observed increase in bacterial adhesion and decrease in invasion, for *S*. Typhimurium SL1344 was due to any residual antibiotics in the macrophages. I quantified cell associated ciprofloxacin concentration in J774 macrophages which were previously incubated with this drug using previously

described methods (Michot et al., 2005). The results (data not shown) revealed that cell associated ciprofloxacin concentration was <100 times less than the MIC of the drug for SL1344. Hence changes in the number of viable bacteria that invaded and adhered to J774 macrophages were not due to cell associated antibiotic.

The innate immune system recognises the presence of bacterial lipopolysaccharide (LPS) through the expression of toll like receptor (TLR). In macrophages, recognition of *S.* Typhimurium is largely mediated by TLR2, TLR4 and TLR5. These TLRs elicit down steam signalling pathways that trigger immune responses such as activation of the inflammatory response. Arpaia et al., (2011) show that TLRs are not only useful in the recognition and attachment to pathogen associated molecular patterns, but are necessary for the survival of *Salmonella* in macrophages. They showed that activation of these TLRs enhances the acidification of the *Salmonella* containing vacuoles. This further induces the expression of SP1-2 genes and translocation of bacterial effector molecules into the macrophage. This results in a more conducive replicative environment in the host and increase in bacterial numbers. Hence, they hypothesized that *Salmonella* exploits host TLR signalling during the infection (Arpaia et al., 2011). It can be extrapolated from their findings that *Salmonella* induces the expression of TLRs which in turn causes more adhesion to the host. It is however unknown if expression of these TLRs are altered in the presence or absence of antibiotics.

The increased numbers of SL1344 *tolC::aph* that invaded the cells was an interesting finding, as it is known that inactivation of *tolC* in *S.* Typhimurium leads to decreased ability to adhere to, and invade host cells (Blair et al., (2009). This suggests that pre-exposure to the antibiotics affected the macrophages in a manner that either altered

macrophage surface structure making SL1344 *tolC::aph* more able to invade, increased survival of this strain intracellularly or increased macrophage ability to phagocytose the *tolC* mutant strain. Although the SL1344 *tolC::aph* is not the strain of focus, the mechanisms behind this observation could be explored in the future for better understanding of the mechanisms behind this observation.

Increased adhesion of bacteria to antibiotic (ciprofloxacin and ceftriaxone) treated macrophages could be potentially beneficial for antibiotic therapy. This implies that more attached bacteria are recognised, internalised and destroyed by immune cells such as macrophages. The decrease in bacterial invasion following antibiotic exposure can be potentially beneficial in the treatment of infections by intracellular bacteria such as *Salmonella* which require intracellular survival in host immune cells. The reduced ability of bacteria to invade macrophage cells treated with azithromycin will result in fewer bacterial reproductive niches within the cell, hence reduced dissemination and spread of the infection to other tissues.

Salmonella invades immune cells via type III secretion systems that deliver bacteria into *Salmonella* containing vacuoles (SCV). This process occurs rapidly and takes only a few minutes. In macrophages, similar processes are involved and increased adhesion on the cell surface may be associated with delayed entry and increased phagocytosis and decreased invasion into SCV (Stones and Krachler, 2015, Finlay and Falkow, 1997). This is the case with C_{max} concentrations of ciprofloxacin, ceftriaxone and azithromycin, where increased bacterial adhesion was observed (Table 4.1). Interestingly, azithromycin at 2mg/ml decreased both adhesion and invasion. However the link between adhesion and invasion and *Salmonella* infection are complex. Kaiser et al, (2014) reported that high-dose ciprofloxacin treatment

efficiently reduced bacterial loads in most organs. However, cecum draining lymph node, the gut tissue, and the spleen retained surviving bacteria in dendritic cells, which remained genetically susceptible to ciprofloxacin, and were sufficient to reinitiate infection after the end of the therapy, displaying an extremely slow growth rate. The slow growth was sufficient to explain recalcitrance to antibiotic treatment and relapse. Targeting the innate immune system increased bacterial clearance (Kaiser et al., 2014). Further, when human monocyte-derived macrophages isolated from peripheral blood of human volunteers were cultured in vitro for macrophage differentiation, infected with *S.* Typhi strains, ceftriaxone inhibited bacterial growth in these cells (Ekinci et al., 2002). Azithromycin affects human M1 macrophage activation, while enhancing M2 differentiation. Importantly azithromycin, down regulated the TLR4 pathway, a response linked to increased *S.* Typhimurium susceptibility in a murine model (Arpaia et al., 2011, Vrancic et al., 2012). These studies support the continued use of ceftriaxone, ciprofloxacin and azithromycin for *S.* Typhimurium infection, but further work on the influence of these drugs on adhesion and invasion, the relation to bacterial stasis and killing, and the maintenance of infection in tissues and antibiotic resistance is required (Wong et al., 2014).

4.7 Key findings:

1. A general pattern of increased bacterial adhesion to antibiotic treated macrophages than in untreated macrophages, especially in the wild type SL1344 strain.

2. Antibiotics at varying concentrations (C_{max} and MIC) have different effects on interaction between *Salmonella* and immune cells.

3. There was no defined trend of effect of antibiotics on wild type virulent *Salmonella* invasion; however for the *tolC* mutant strain, there was increased bacterial invasion to macrophages pre-exposed to all antibiotics except azithromycin.

4. Interaction between bacteria and macrophages pre-treated with antibiotics is strain dependent as different stains adhered to and invaded differently even when exposed to the same concentration of antibiotics.

5. Similar effect of increase in adhesion of virulent *Salmonella* was observed in both cell line and primary mouse macrophages exposed to antibiotics.

4.8 Further work

My data showed that there was increased bacterial adhesion to macrophages pre-treated with ciprofloxacin and ceftriaxone. Investigating the effect of antibiotics on TLR signalling involved with recognising and attaching to conserved bacterial molecular patterns therefore forms the basis of the next chapter.

CHAPTER FIVE

5 The effect of antibiotic pre-treatment on the functions of human immune cells.

5.1 Background.

The results presented in Chapter 4 (Sections 4.4 and 4.5) illustrate that adhesion to, and invasion of, wild type *Salmonella* SL1344 and three isogenic strains to antibiotic pre-treated immune cells of **murine** origin; J774 macrophages (cell line) and bone marrow derived macrophages (BMDMs, primary cells), is altered after antibiotic exposure. My experiments showed that ciprofloxacin and ceftriaxone most frequently affected bacterial adhesion to both cell types. Hence, in this chapter, the effects of these two antibiotics on bacterial adhesion to, and invasion of, **human** immune cells; THP-1 and monocyte derived macrophages (MDMs), were considered. THP-1 cells were chosen because they are a monocytic cell line of human origin that can be differentiated into mature macrophages in the presence of phorbol 12-mistate 13-acetate (PMA). Hence, they are suitable for comparison with primary cell i.e. monocyte derived macrophages from peripheral whole blood.

Further, the effects of antibiotic pre-exposure on neutrophils (from **human** peripheral whole blood) functions *in vitro* were evaluated. Phagocytosis and oxidative burst are key elements of neutrophil activity against bacteria. The effect of the antibiotics (excluding streptomycin) used in the preceding chapters on neutrophil phagocytosis and oxidative burst was analysed using flow cytometry, while neutrophil killing was assessed by determining the viability of bacteria, post incubation with antibiotic treated neutrophils.

5.2 Hypothesis.

To build upon data obtained in Chapter 4, it was hypothesized that pre-exposure of human macrophages to ciprofloxacin and ceftriaxone would affect bacterial adhesion and invasion in the same manner as for murine macrophages. It was also hypothesized that antibiotic pre-treatment makes neutrophils more effective at generating oxidative burst, phagocytosis and killing of bacteria.

5.3 Aims.

1. To determine the adhesion to and invasion of, *Salmonella* to antibiotic pre-exposed THP-1 and primary human macrophages.
2. To investigate the effects of pre-exposure to antibiotics (C_{max}) on the activities of human peripheral neutrophils *in vitro*.

5.4 Adhesion to, and invasion of, *Salmonella* to antibiotic treated human THP-1 macrophages.

Data shown in Figure 5.1 show the results obtained for invasion and adhesion of *S.* Typhimurium SL1344 to THP-1 macrophages pre-treated with ciprofloxacin and ceftriaxone at concentrations mimicking the human C_{max} for 2 hours. Pre-exposure of THP-1 macrophages to ciprofloxacin and ceftriaxone (represented as CIP and CRO (internationally recognised abbreviations for these antibiotics) in Figures 5.1 and 5.2) did not significantly alter bacterial adhesion compared to non-treated controls (Figure 5.1a).

Similarly, there was no significant difference in the CFU/ml of bacteria that adhered to, or invaded, ciprofloxacin or ceftriaxone treated human MDMs (Figure 5.2). Interestingly, there was a decrease in the ability of SL1344 to invade antibiotic

treated THP-1 macrophages (~10^2 CFU/ml of bacteria; Figure 5.1b) compared to human MDMs (~10^4 CFU/ml of bacteria; Figure 5.2b) where bacterial invasion of antibiotic treated macrophages was not altered.

Figure 5.1 Adhesion to, and invasion of, *S.* Typhimurium SL1344 to antibiotic treated human THP-1 macrophages.

a.
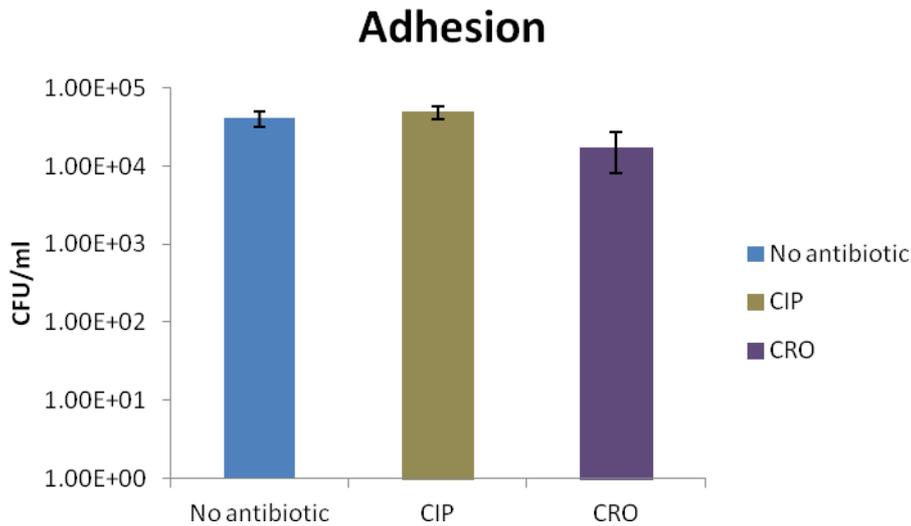
b.
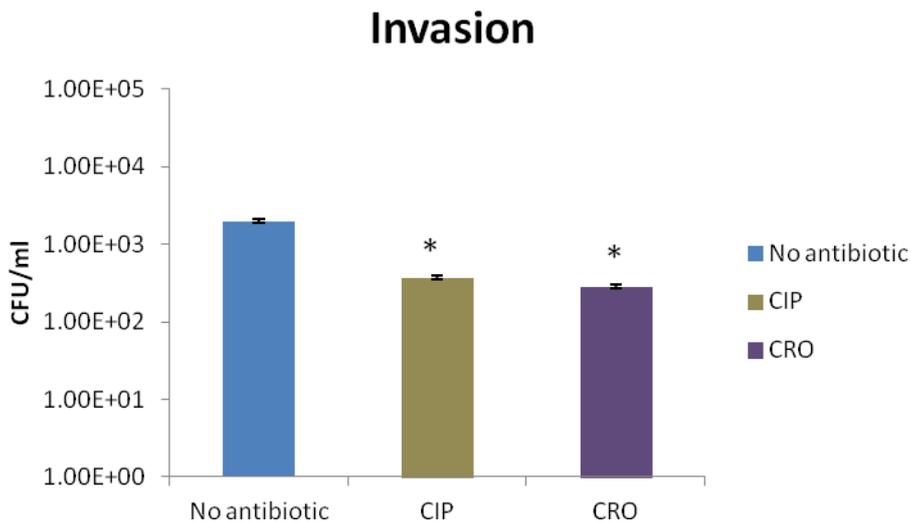
The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The blue, green and purple bars represent colony forming units of bacteria that adhered to or invaded untreated, ciprofloxacin and ceftriaxone treated macrophages respectively. A Student's T-test was carried out to compare viable counts between treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.2 Adhesion to, and invasion of, *S.* Typhimurium SL1344 in antibiotic treated human MDMs.

a.
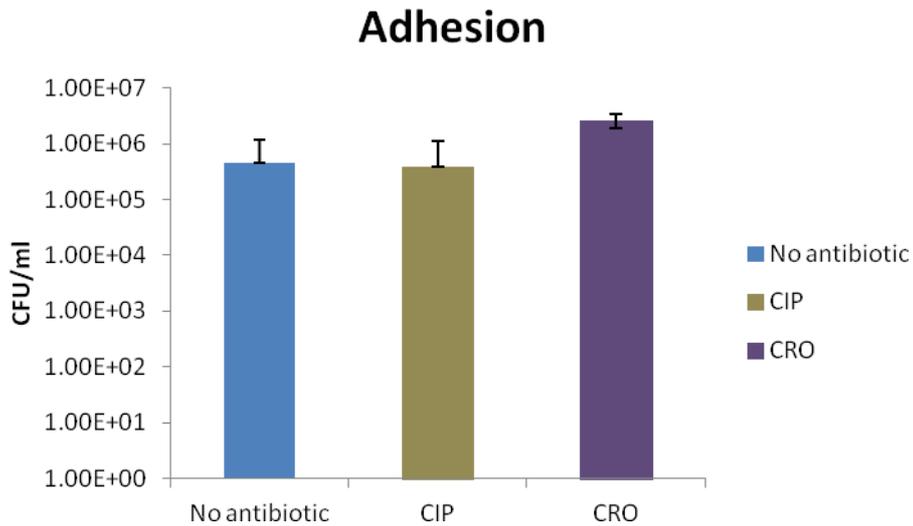
b.
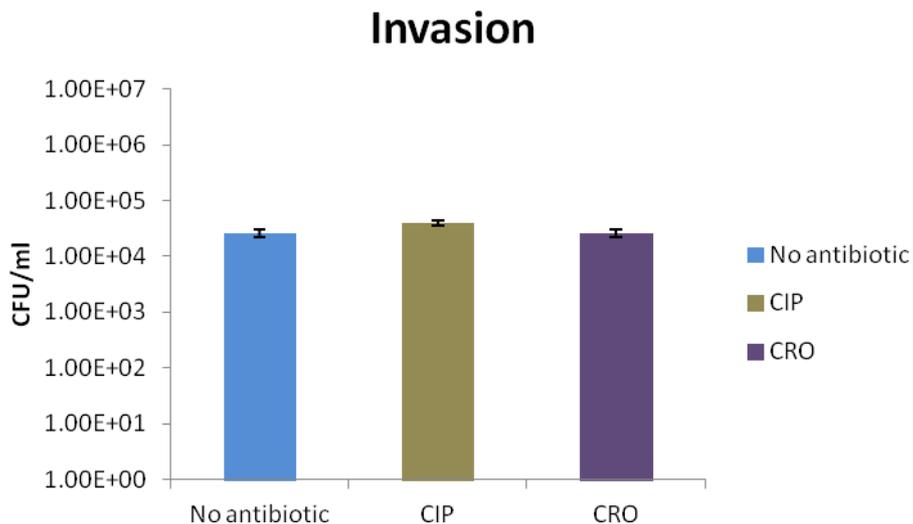
The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The blue, green and purple bars represent colony forming units of bacteria that adhered to or invaded untreated, ciprofloxacin and ceftriaxone treated macrophages respectively. A Student's T-test was carried out to compare viable counts between treated and untreated macrophages for each strain. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

5.5   Effect of antibiotics on killing of opsonised wild type *S.* Typhimurium SL1344 and SL1344 *tolC::aph* by human peripheral blood neutrophils.

Neutrophils were isolated from peripheral whole blood of healthy volunteers and incubated with $1/_{10}$ x MIC, MIC and C_{max} concentrations of antibiotics for 15 minutes at 37⁰C prior to addition of bacteria.

Pre-exposure of neutrophils to 0.0015 ($1/_{10}$ x MIC) and 0.015 µg/ml (MIC) of ciprofloxacin for 30 and 60 minutes of incubation did not significantly affect their ability to kill *S.* Typhimurium SL1344. However, there was a slight but not significant decrease in the viability of SL1344 retrieved after 60 minutes of incubation with neutrophils pre-treated with 5 µg/ml (C_{max}) of ciprofloxacin (Figure 5.3). Similar to SL1344 (Figure 5.3a), when neutrophils were pre-treated with 5 µg/ml of ciprofloxacin, there was a slight but not significant decrease in the numbers of viable SL1344 *tolC::aph* 60 minutes post incubation (Figure 5.3b).

When neutrophils were pre-treated with 0.8 µg/ml of ceftriaxone ($1/_{10}$ x MIC for SL1344) compared to untreated neutrophils, there was no difference in their ability to kill SL1344 or SL13444 *tolC::aph* at 30 and 60 minutes incubation. However, in the presence of 8 and 82 µg/ml of ceftriaxone after 30 and 60 minutes incubation, neutrophils killed significantly more SL1344 than untreated neutrophils (Figure 5.4a). Neutrophils pre-treated with 8 and 82 µg/ml of ceftriaxone also killed significantly more SL1344 *tolC::aph* than untreated neutrophils at both time points. There were no differences at either time points in the numbers of SL1344 *tolC::aph* killed by neutrophils pre-treated with 0.8 µg/ml of ceftriaxone (Figure 5.4b). Neutrophils killing were unaltered by pre-treatment with tetracycline, azithromycin or streptomycin (Figures 5.5 – 5.7).

150

Figure 5.3 Viable count of *S.* Typhimurium SL1344 and SL1344 *tolC::aph* after incubation with ciprofloxacin pre-treated neutrophils.
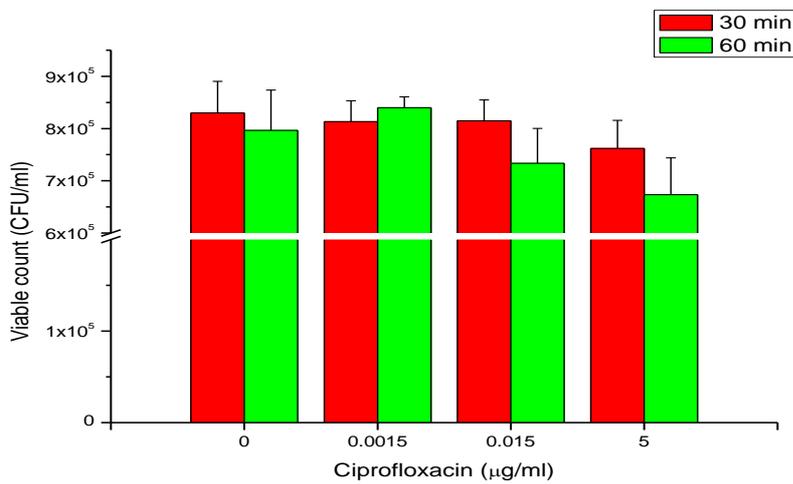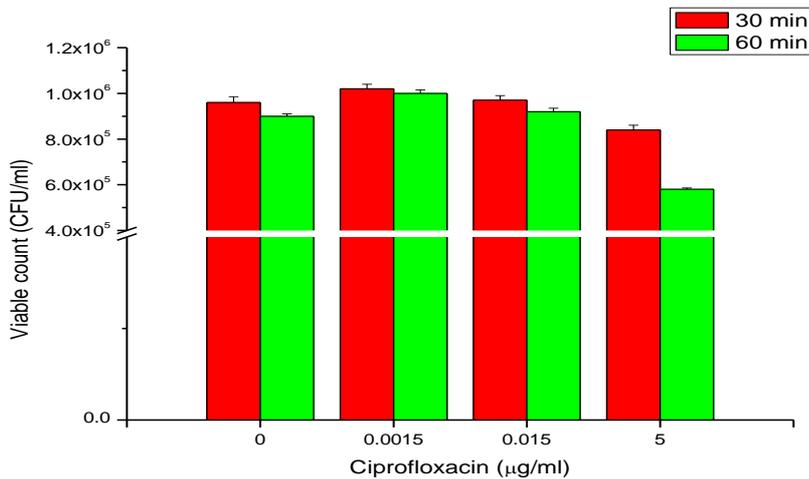
a. SL1344

b. SL1344 *tolC::aph*

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The "0" represents no antibiotic treatment. The red bars indicate bacteria viability after 30 minutes while green bars represent bacteria viability after 60 minutes. Statistical significance was analysed by a two-tailed Student's T-test. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant ($p>0.05$), n=3.

Figure 5.4 Viable count of *S.* Typhimurium SL1344 and SL1344 *tolC::aph* after incubation with ceftriaxone pre-treated neutrophils.
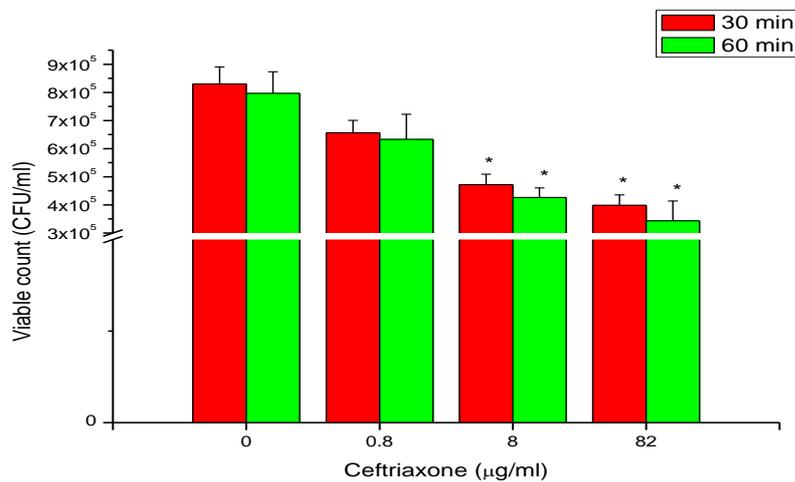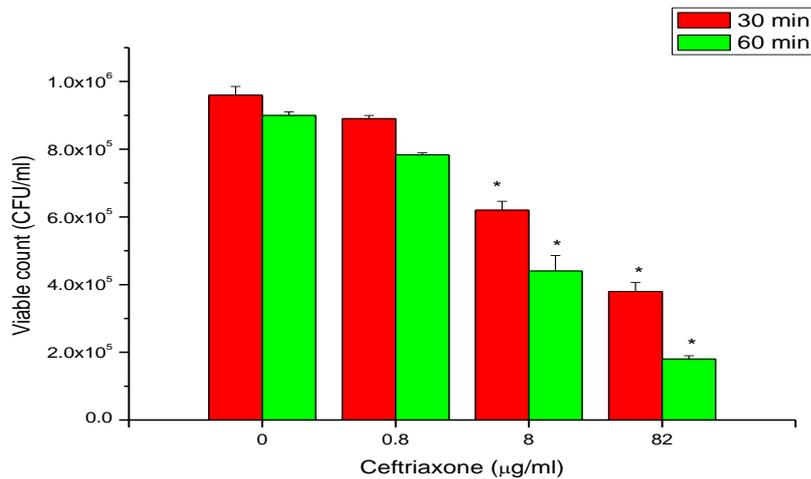
a. SL1344

b. SL1344 *tolC::aph*

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The "0" represents no antibiotic treatment. The red bars indicate bacteria viability after 30 minutes while green bars represent bacteria viability after 60 minutes. Statistical significance was analysed by a two-tailed Student's T-test. The * indicates a significant difference ($p < 0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.5 Viable count of *S.* Typhimurium SL1344 and SL1344 *tolC::aph* after incubation with tetracycline pre-treated neutrophils.
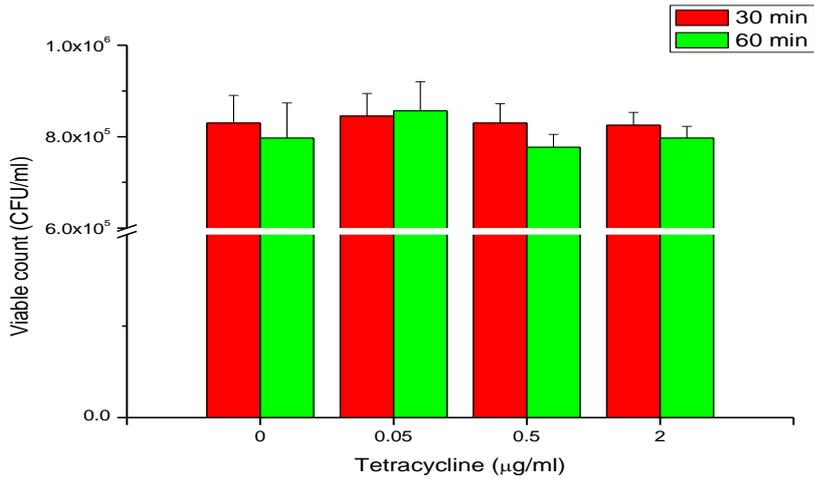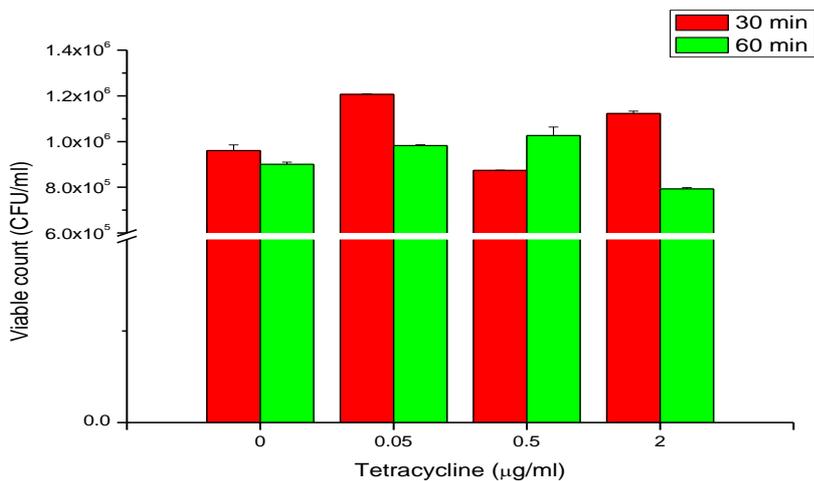
a. SL1344

b. SL1344 *tolC::aph*

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The "0" represents no antibiotic treatment. The red bars indicate bacteria viability after 30 minutes while green bars represent bacteria viability after 60 minutes. Statistical significance was analysed by a two-tailed Student's T-test. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.6 Viable count of *S*. Typhimurium SL1344 and SL1344 *tolC::aph* after incubation with azithromycin pre-treated neutrophils.
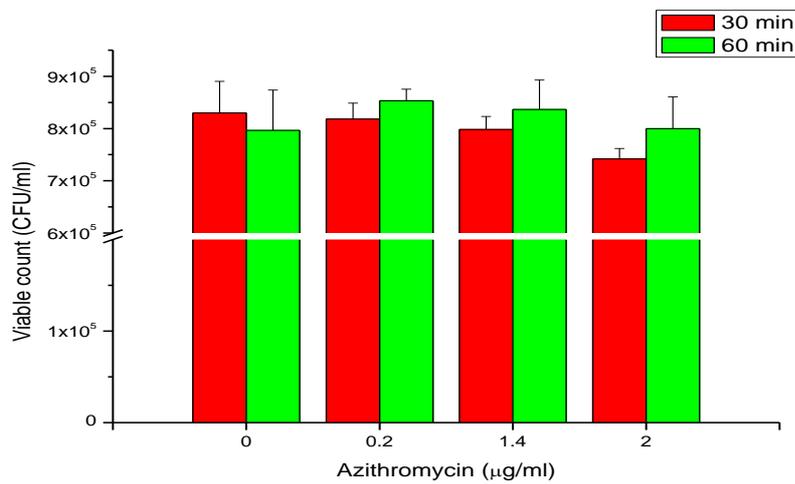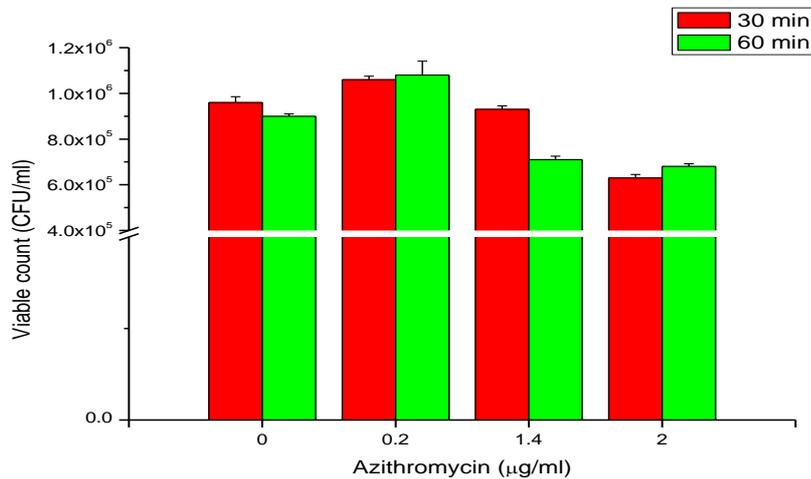
a. SL1344

b. SL1344 *tolC::aph*

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The "0" represents no antibiotic treatment. The red bars indicate bacteria viability after 30 minutes while green bars represent bacteria viability after 60 minutes. Statistical significance was analysed by a two-tailed Student's T-test. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.7 Viable count of *S*. Typhimurium SL1344 and SL1344 *tolC::aph* after incubation with streptomycin pre-treated neutrophils.
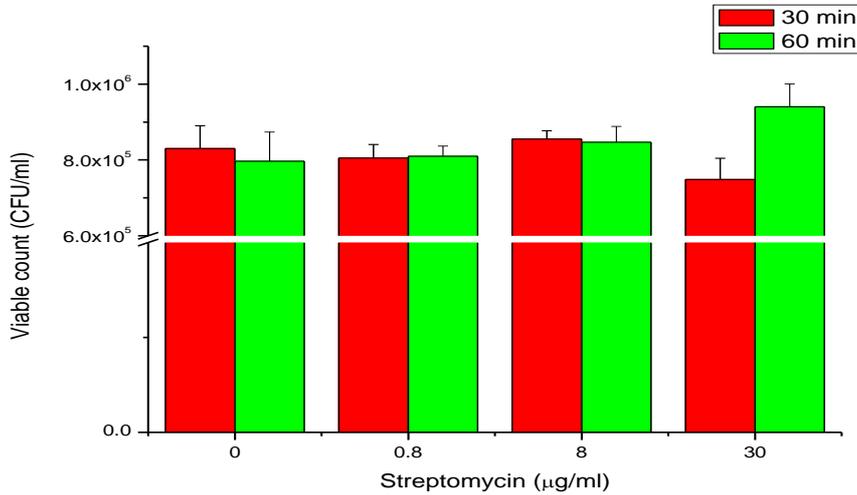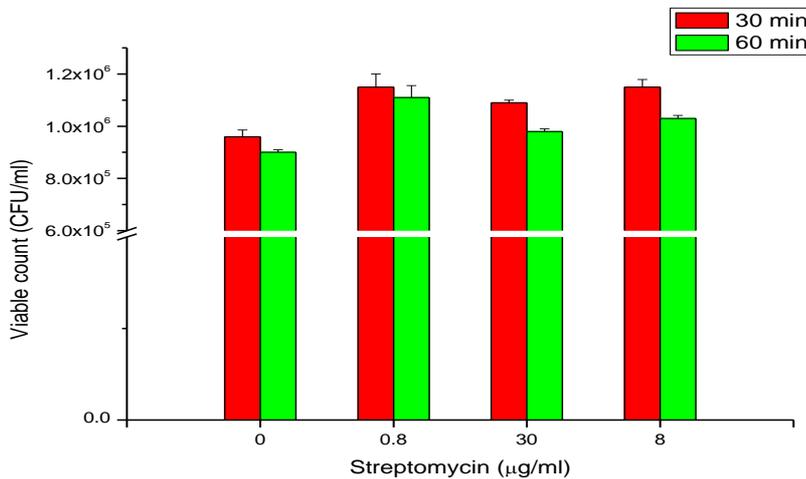
a. SL1344

b. SL1344 *tolC::aph*

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). The "0" represents no antibiotic treatment. The red bars indicate bacteria viability after 30 minutes while green bars represent bacteria viability after 60 minutes. Statistical significance was analysed by a two-tailed Student's T-test. The * indicates a significant difference (*p*<0.05), all other comparisons were considered non-significant, n=3.

5.6 The effect of C_{max} concentrations of antibiotics on phagocytosis and oxidative burst activity of neutrophils.

Phagocytic capacity of neutrophils was assessed using FACS by measuring the uptake of FITC labelled bacteria, while oxidative burst after phagocytosis of bacteria was estimated by the amount of fluorescence produced following oxidation of dihydrorhodamine (DHR)-123.

Ciprofloxacin

When neutrophils were pre-exposed to C_{max} concentration of ciprofloxacin (5 µg/ml), there was a significant decrease in their ability to phagocytose bacteria (Figure 5.8a). However, oxidative burst activity was unaltered in neutrophils pre-treated with 5 µg/ml of ciprofloxacin and stimulated with either fMLP, PMA or bacteria (Figure 5.8b)

Ceftriaxone

Pre-treatment of neutrophils with 82 µg/ml of ceftriaxone (C_{max}) also did not result in any significant changes in the phagocytic ability of these cells (Figure 5.9a). However, compared to untreated neutrophils exposure of neutrophils to the same concentration of ceftriaxone resulted in a significant increase in oxidative burst activity (Figure 5.9b).

Figure 5.8 Effect of ciprofloxacin (5 μg/ml; C$_{max}$) on phagocytosis and oxidative burst of neutrophils.
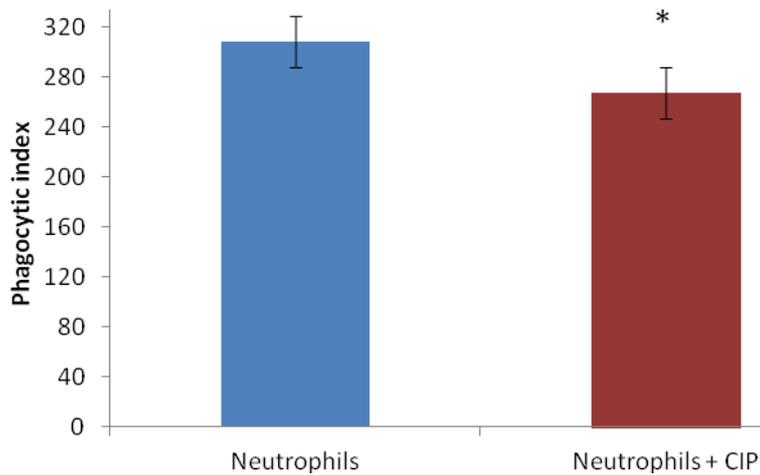
a. Phagocytosis
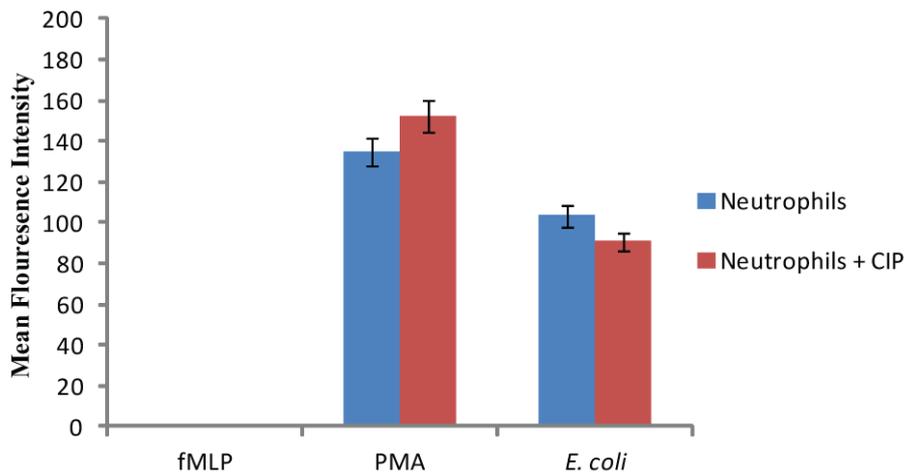
b. Oxidative burst

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). Phagocytosis or oxidative burst in CIP treated neutrophils (red bars) was compared against non-antibiotic treated neutrophils (blue bars). The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.9 Effect of ceftriaxone (82 µg/ml; C_{max}) on phagocytosis and oxidative burst of neutrophils
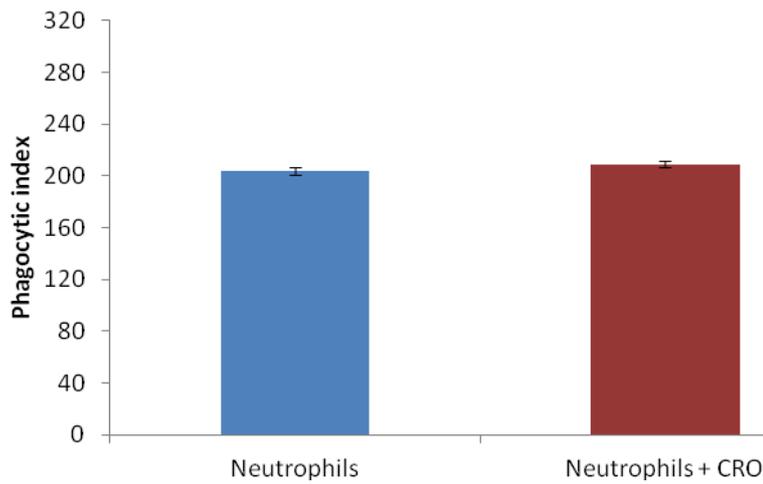
a. Phagocytosis
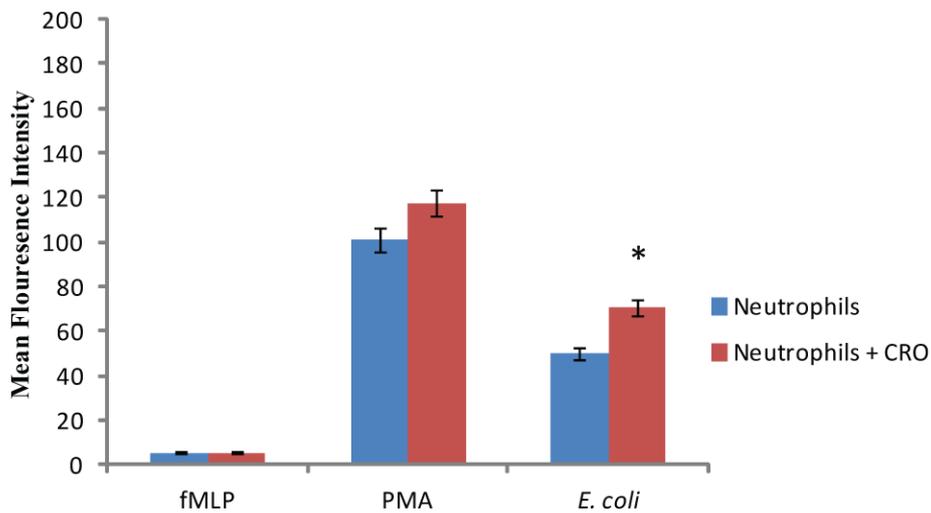
b. Oxidative burst

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). Phagocytosis or oxidative burst in CRO treated neutrophils (red bars) was compared against non-antibiotic treated neutrophils (blue bars). The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Tetracycline

There was no significant difference between the phagocytic abilitiy of neutrophils pre-exposed to 2 µg/ml of tetracycline compared to untreated neutrophils (Figure 5.10a). Further, there was no significant difference in the oxidative burst in tetracycline treated or untreated neutrophils stimulated with fMLP or bacteria. However, exposure to tetracycline reduced oxidative burst of neutrophils stimulated with PMA was (Figure 5.10b).

Azithromycin

Pre-exposure of neutrophils to 1.4 µg/ml of azithromycin had no significant affect on phagocytosis compared to untreated neutrophils (Figure 5.11a). Similarly, the oxidative burst in azithromycin pre-treated neutrophils stimulated with fMLP, PMA or bacteria was not significantly different from non-antibiotic treated neutrophils (Figure 5.11b).

Figure 5.10 Effect of tetracycline (2µg/ml; C_{max}) on phagocytosis and oxidative burst of neutrophils
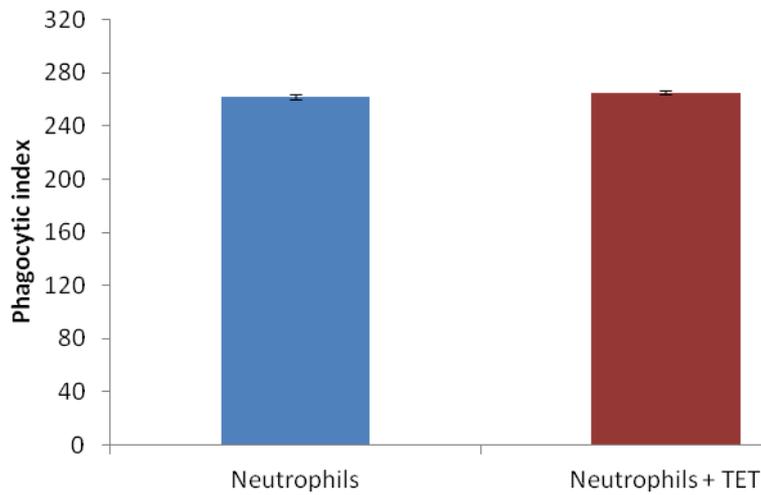
a. Phagocytosis
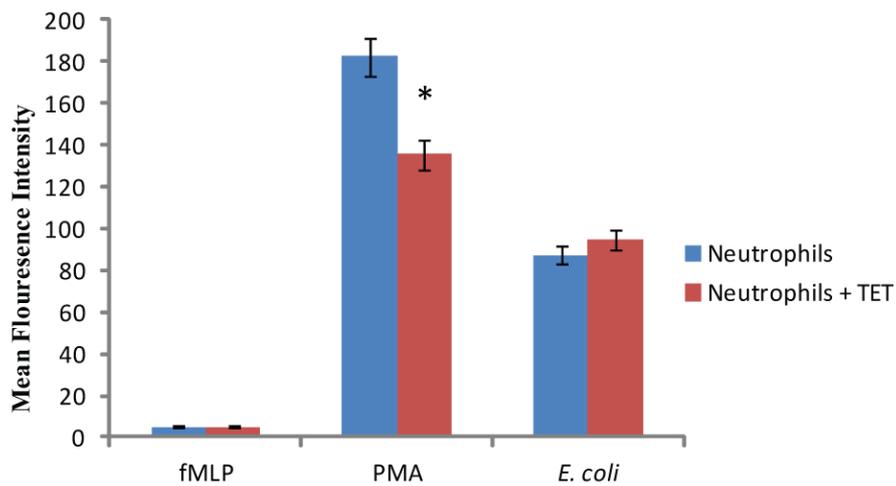
b. Oxidative burst

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). Phagocytosis or oxidative burst in TET treated neutrophils (red bars) was compared against non-antibiotic treated neutrophils (blue bars). The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 5.11 Effect of azithromycin (1.4 µg/ml; C_{max}) on phagocytosis and oxidative burst of neutrophils.
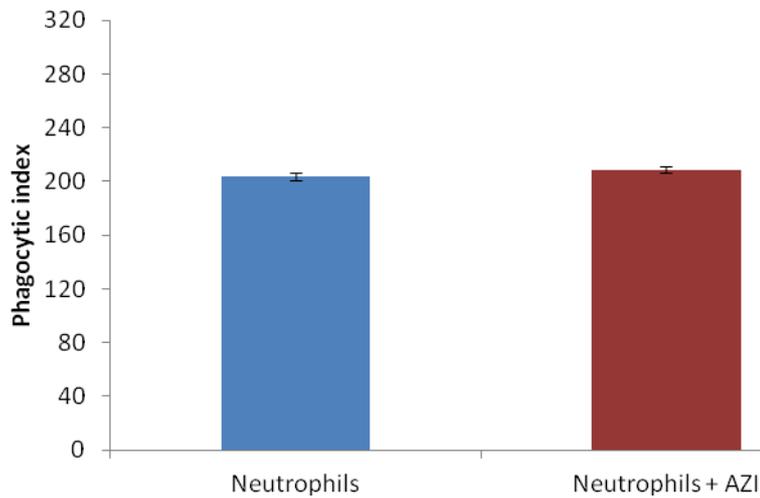
a. Phagocytosis
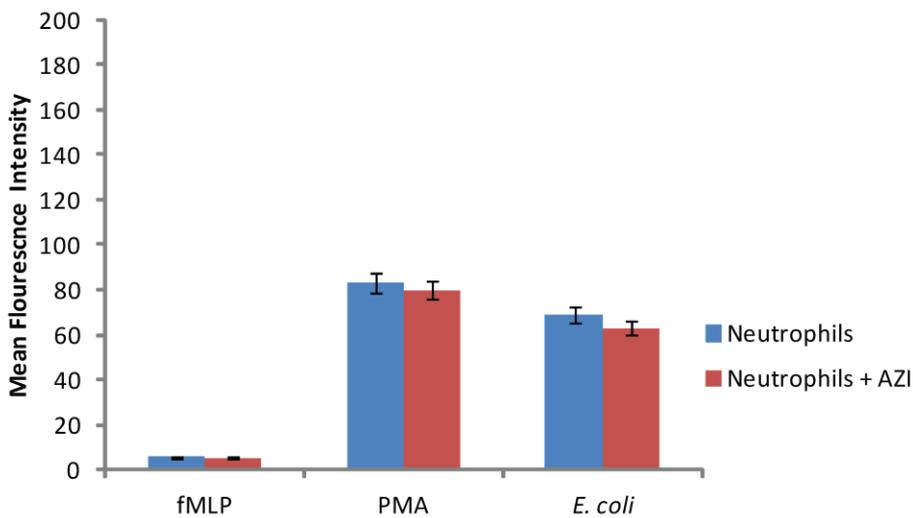
b. Oxidative burst

The data presented in the charts are the means of three individual experiments performed in triplicate (+/- SD). Phagocytosis or oxidative burst in AZI treated neutrophils (red bars) was compared against non-antibiotic treated neutrophils (blue bars). The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

5.7 Summary of the effects antibiotics on adhesion to, and invasion of, *Salmonella* in human macrophages, and neutrophil functions.

Compared to non-treated macrophages, adhesion of *Salmonella* was not altered in THP-1 and MDM macrophages pre-exposed to 5 µg/ml of ciprofloxacin (C_{max}). Similarly, pre-exposure of macrophages to 82 µg/ml of ceftriaxone did not alter adhesion of *Salmonella*. However, there was significant decrease in invasion of *Salmonella* to THP-1 and MDMs pre-exposed to ceftraixone (Table 5.1).

Phagocytosis and killing was significantly higher following neutrophil pre-exposure to ciprofloxacin but not in neutrophils pre-exposed to ceftriaxone, tatracycline or azithromycin.

Table 5.1 Summary table of data for adhesion to, and invasion of *Salmonella* in antibiotic pre-treated human macrophages

	THP-1s		MDMs	
Antibiotic treatment (C_{max})	Adhesion	Invasion	Adhesion	Invasion
Ciprofloxacin (5 µg/ml)	-	↓	-	-
Ceftriaxone (82 µg/ml)	-	↓	-	-

Table 5.2 Summary table of data for the effect of antibiotics on neutrophil phagocytosis, oxidative burst and killing

	Neutrophil function		
Antibiotic treatment (C_{max})	Phagocytosis	Oxidative burst	Killing
Ciprofloxacin (5 µg/ml)	↓	-	↑ (L109, after 60 minutes)
Ceftriaxone (82 µg/ml)	-	↑	-
Tetracycline (2 µg/ml)	-	-	-
Azithromycin (1.4 µg/ml)	-	-	-

5.8 Discussion

Further to the experiments described in Chapter 4, the experiments in this chapter sought to determine whether antibiotic pre-treatment of human THP-1s and MDMs altered bacterial adhesion to, and invasion of, these cells. Data obtained from murine macrophages (J774 and BMDMs) showed that pre-exposure to maximum serum levels (C_{max}) of ceftriaxone and ceftriaxone for two hours increased bacterial adhesion to these cells. However, the same was not found with the human macrophages where antibiotics did not significantly alter the adhesion to, or invasion of SL1344, in human derived macrophages (MDMs).

Interestingly, SL1344 was less able to invade antibiotic treated THP-1 macrophages in comparison to the MDMs. This observation could be because THP-1 cells are an immortalised cell line or to changes in cell morphology of the cells during maturation with PMA, making it more difficult for bacteria to invade. In addition to changes in cell morphology, it has been shown that treatment of mature THP-1 cells with PMA, induces increased phagocytic ability of the cell, as well as cytokine expression similar to that of human MDMs during TLR responses (Daigneault et al., 2010). It is possible that pre-treatment of THP-1 cells with ciprofloxacin and ceftriaxone cause intracellular changes in the macrophages, making them less tolerable for SL1344 survival, hence the lower number of bacteria retrieved from invasion assays. To confirm this, it would be worthwhile investigating whether exposure to antibiotics or intracellular accumulation of antibiotics causes changes in the internal organelles of the cells, and subsequently the survival of *Salmonella*.

Although bacterial adhesion to, and invasion of, antibiotic treated THP-1 and MDM is not extensively discussed in the literature, infection assays by Carryn et al., (2002)

showed that THP-1 cells exposed to ciprofloxacin had reduced activity against intracellular bacteria (*Listeria monocytogenes*) in comparison to ciprofloxacin only in liquid medium (Carryn et al., 2002). The effect of antibiotics on other processes such as cytokine production and phagocytosis has been outlined. In 1991, Simon et al., showed that exposure of THP-1 cells to tissue culture medium (RPMI) containing 5µg/ml of ciprofloxacin did not greatly alter the level of TNFα secretion compared to β-lactam antibiotics such as ceftazidime and cefotaxime (Simon et al., 1991).

Neutrophils are a crucial component of the host's immune response and form the first line of defence against invading organisms. Their main functions include ingestion and killing of bacteria and other pathogens such as fungi yeast and parasites. During infection, neutrophils play a key role because of their ability to phagocytose bacteria as well as generate reactive oxygen species. Neutrophils also release cytokines and chemokines such as IL-17 and CXCL8 which attract other immune cells to the sites of infection (Khader and Cooper, 2008). In addition, neutrophils extrude extracellular fibrillary networks known as neutrophil extracellular traps (NETs), which capture microorganisms and facilitate their interaction with neutrophils (Zawrotniak and Rapala-Kozik, 2013).

The interaction between neutrophils and the antibacterial agents may affect the fate of bacteria ingested by immune cells, especially by the phagocytes. This interaction has the potential to be beneficial in the treatment of infectious diseases. However, the effect of antibiotics on neutrophil function is the subject of conflicting reports.

While my data shows no alteration in the ability of ciprofloxacin pre-treated bacteria to kill wild type *S*. Typhimurium, Cacchillo and Walters (2002) however demonstrated that in neutrophils pre-exposed to ciprofloxacin at 0.5 µg/ml, there was enhanced

killing of *Actinobaccillus actinomycetemcomitans* (a pathogen implicated in aggressive periodontitis), compared to non-treated neutrophils. *In vitro* studies by Canton et al., (1999) also showed that at a ciprofloxacin concentration of 1µg/ml, 90% of phagocytosed *S.* Typhi were killed by ciprofloxacin treated neutrophils after 30 min (Canton et al., 1999). At the same concentration of ciprofloxacin in a similar study, 90% of phagocytosed *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Corynebacterium* group D2 was killed by ciprofloxacin treated neutrophils (Peman et al., 1994). Again, *in vivo* studies by Easmon et al., (1886) showed that ciprofloxacin was successful in treating murine systemic infection with *S.* Typhimurium, and hindered the progression of the disease in mice lacking natural immunity (Easmon et al., 1986).

Further, Boogearts et al, (1986) investigated the immunomodulatory effect of four fluoroquinolones (ciprofloxacin, ofloxacin, pefloxacin and norfloxacin). Ciprofloxacin did not induce any significant effect on neutrophil functions at concentrations of 0.1, 1. 5 and 10 µg/ml (Boogaerts et al., 1986). Some authors argued that the antibiotic effect is not directly on the immune responses but on the bacteria, making them more susceptible to killing by the immune system (Forsgren and Bergkvist, 1985). It is also reported that ciprofloxacin improves neutrophil functions such as intracellular killing oxidative burst activity in both healthy volunteers and in type 2 diabetes mellitus patients with deteriorating immune system (Rayaman et al., 2013). The mechanism behind this is not clearly understood.

From data presented in this thesis, the decreased phagocytosis by neutrophils after ciprofloxacin exposure could be due to the potent effect of this antibiotic on bacteria viability as seen at the 2 hour time point (Figure 3.1), while the increased oxidative

burst following ceftriaxone treatment during an infection enhances the antibacterial oxidative killing mechanisms of neutrophils. Together these findings support a synergistic effect of antibiotic treatment of bacteria and immune cells in increasing bacterial killing and potentially reducing the spread of infection and are potentially useful in clinical conditions in which neutrophil function is compromised. However, the complex nature of such responses is clear.

Carreer et al., (1998) investigated the effect of beta-lactams (ceftazidime, ceftriaxone and cefuroxime) on stimulated human polymorphonuclear neutrophils and showed that none of these antibiotics had an effect on the oxidative mechanism in neutrophils (Carreer et al., 1998). Further, the effects of ceftriaxone and ceftodizine on neutrophils from patients with severe bacterial infections show that daily administration of 50mg of ceftriaxone per kg for 10 days improved phagocytic functions compared to pre-therapy. Although the underlying factors for this effect were not stated, it was suggested from this publication that the antibiotics enhance the reactive oxygen production capacity of neutrophils. They suggest that in treatment of immunocompromised patients with severe bacterial infections, antibiotics that enhance the phagocytic activity of neutrophils should be used (Wenisch et al., 1995).

In neutrophils, tetracyclines inhibit the synthesis of reactive oxygen species (Minic et al., 2009). Hence, tetracyclines are anti-inflammatory and immunomodulatory (Kuzin et al., 2001). Their ability to suppress neutrophil oxidative activities is because they are able to cross the cell's plasma membrane (Gabler, 1991).The immunomodulatory properties of tetracycline include inhibition of host-derived (neutrophil) collagenases and other matrix metalloproteinases (Suomalainen et al., 1992). In early studies,

where *in vivo* and *in vitro* effects of tetracycline on human monocytes and lymphocytes were tested, no significant changes in monocyte functions was observed. However, this was refuted by subsequent work by Walters (2006) where it was shown that despite its low absorbability and affinity, tetracycline at clinically relevant levels (1-4µg/ml) accumulate within neutrophils at relatively high concentrations. Further, it was proposed that this accumulation would be beneficial in enhancing the killing of intracellular bacteria. (Walters, 2006).

To understand how azithromycin activities affect the functions of neutrophils, a study involving the administration of a 3-day standard antibacterial dose (500mg) of azithromycin on healthy volunteers was carried out (Culic et al., 2002). They tested the effects of azithromycin on neutrophil functions such as neutrophil oxidative burst, apoptosis, degranulation, and circulating chemokines. These experiments were carried out 2.5h, 24h and 28 days post antibiotic treatment.

 In contrast to their work, incubation of neutrophils with azithromycin did not affect either phagocytosis or oxidative burst in my study. My data did not show any significant difference between bacterial killing in azithromycin pre-treated and non-azithromycin treated neutrophils. By contrast, and using similar neutrophil killing experiments, it was shown that neutrophils pre-treated with macrolide antibiotic, clarithromycin (a 14- membered ring macrolide) killed significantly more bacteria than non-treated neutrophils (Iskandar and Walters, 2010). Recent studies have shown that pre-treatment of mice with azithromycin for two hours before challenge with lipopolysaccharide, reduced neutrophil numbers and inflammatory markers in bronchial lavage fluid, and induced tolerance to endotoxin challenge in BALB/cJ mice. (Bosnar et al., 2009, Bosnar et al., 2013, Bosnar et al., 2011). The authors

identified mechanisms such as macrophage mediated inhibition of activator protein-1 (AP-1) and IL-1β. This is at variance with data presented in this thesis. The reasons for the discrepancies may be that Bosnar and colleagues used animal models in their experiments, while tissue culture models of infection was used in this study. Also, although the mice were exposed to azithromycin 2 hours prior to LPS challenge, there were still circulating levels of antibiotics. Reduced cell numbers in infected tissue could prevent excessive tissue damage, in neutrophil-dominated inflammatory diseases (Erakovic Haber et al., 2014). However, this does not rule out an effect of azithromycin on the function of the neutrophils that enter tissues.

It is important to note that the half-life of circulating neutrophils in circulating blood is 8- 10 hours (Akgul et al., 2001) and neutrophil life span is 5.4 days (Pillay et al., 2010). Therefore, data by Culic et al., (2002) represents a cumulative activity since the neutrophils are produced, circulate, undergo antimicrobial functions and eventually apoptosed. Therefore, measurement of these parameters at 24h and 28 days post antibiotic administration may not offer an ideal interpretation neutrophil function in the presence of azithromycin.

5.9 Key points:

- Of the four antibiotics tested, only ciprofloxacin reduced the capacity of neutrophils to phagocytose bacteria.

- Phagocytosis was not altered in the presence of ceftriaxone, tetracycline or azithromycin.

- There was an increased oxidative burst in neutrophils pre-treated with ceftriaxone but not ciprofloxacin, tetracycline or azithromycin.

168

- Neutrophils pre-treated with ceftriaxone at all concentrations above 8 µg/ml showed significant reductions in the viability of bacterial up to 60 minutes after bacteria incubation.

- Compared to untreated neutrophils, following ciprofloxacin, tetracycline, streptomycin and azithromycin pre-treatment, the ability of neutrophils to kill *Salmonella* was not altered.

5.10 Future work

Further to these experiments, future work should include;

- Identifying the sub-cellular locations of ciprofloxacin and ceftriaxone in neutrophils using confocal microscopy with fluorescently labelled antibiotics (Denamur et al., 2011), and if accumulation of these drugs affect neutrophil metabolism.

- Exploring which mechanisms (such as inhibition of protein kinase C (PKC)) in neutrophils are altered following antibiotic exposure, leading to decreased phagocytosis (in ciprofloxacin exposed neutrophils) and increased oxidative burst (in ceftriaxone exposed neutrophils). PKC activity in cytosolic and membrane fractions of antibiotic treated neutrophils can be measured using a PKC-selective peptide substrate which has been previously described (Chakravarthy et al., 1991).

CHAPTER SIX

6 Effect of antibiotic pre-treatment on TLR gene expression in J774 macrophages.

6.1 Introduction.

In Chapter 4, it was shown that there was increased adhesion of *Salmonella* to ciprofloxacin and ceftriaxone pre-treated murine cell line (J774 macrophages) and primary murine bone marrow derived macrophages (BMDM) compare to untreated macrophages. The mechanism of increased adhesion of *Salmonella* to antibiotic treated macrophages is unknown.

Immune cells such as macrophages and neutrophils recognize and bind to conserved microbial patterns e.g. lipopolysaccharide (LPS) using Pathogen Recognition Receptors (PRR) (Hayashi et al., 2003). Toll like receptors (TLR), a family of the PRR, are primary sensors of pathogens and are the most studied of the pathogen recognition receptors (Creagh and O'Neill, 2006). Each PRR binds to a specific ligand, initiates an innate immune response and subsequently activates an adaptive immune response (Lavelle et al., 2010). TLR4 for instance is involved in recognition of endotoxin released from Gram-negative bacteria such as *Salmonella* (Arpaia et al., 2011, Broz et al., 2012); it also triggers phagocytosis of pathogens, as well as eliciting the induction of inflammatory cytokines. The NOD like receptors (NLR) are another family of PRR and sense conserved microbial patterns inside immune cells. The NLR are made up of five proteins; NOD1, NOD2, NLRC3, NLRC4 and NLRC5 (Antosz and Osiak, 2013). Both NLR and TLR play important roles in the immune response to microbial infections.

It is known that some antibiotics such as ciprofloxacin, ceftriaxone, tetracycline and azithromycin have immunomodulatory properties that affect the immune response to

bacteria or bacterial ligands such as LPS, (Katsuno et al., 2006, Bode et al., 2014, Tai et al., 2013). However little is known about the underlying mechanism by which antibiotics affect TLR signaling and function in response to *Salmonella*. This chapter examined the effects of ciprofloxacin and ceftriaxone at their respective maximum serum concentrations (C_{max}) on the expression of genes on the TLR signaling pathway in response to *Salmonella*. Antibiotics were selected for further study in this chapter based on those with the most observed effects in prior assays, as described in previous chapters of this thesis.

6.2 Hypothesis.

It was hypothesised that pre-exposure to antibiotics increases the expression of TLR leading to increased recognition of, and binding to the *Salmonella* LPS, hence increased adhesion. Since TLR signaling also affects the induction of cytokines, the production of cytokines by antibiotic pre-treated macrophages was also investigated.

6.3 Aims.

- To investigate the mRNA expression of genes in the TLR pathway in the presence or absence of ciprofloxacin and ceftriaxone.
- To determine if mRNA expression of genes in the TLR signaling pathway translated to protein expression using ELISA.

6.4 Results

6.4.1 Assessment of RNA purity

Using good quality RNA is crucial in obtaining reliable gene expression data. For this reason, RNA integrity was assessed spectrophotometrically via Nanodrop and by

visual inspection of the 18S and 28S ribosomal RNA bands via denaturing agarose gel electrophoresis (Figure 6.1 and 2).

Intact non-degraded RNA is represented by clear distinct 28S and 18S RNA bands after gel electrophoresis, ensuring that pre-exposure to antibiotics (ciprofloxacin and ceftriaxone) or *Salmonella* did not affect J774 macrophage RNA integrity.

6.4.2 RT-PCR analysis of gene expression in the TLR signaling J774 macrophages following antibiotic treatment.

To determine the effect of antibiotics on TLR signaling, in three separate experiments, J774 macrophages were incubated with or without maximum serum concentrations of antibiotics (ciprofloxacin, 5 μg/ml; ceftriaxone, 8 μg/ml) for 2 hours prior to infection or no infection with *Salmonella*. The expression of eighty-four genes in the TLR signaling pathway was monitored by RT-PCR after 2 hours incubation. The tested antibiotics conferred differential TLR gene expression. The magnitude of difference between gene expression in antibiotic treated and untreated macrophages was measured in fold change.

The online RT^2 profiler PCR Array Data Analysis Software v3.5 by SABiosciences was used to evaluate fold change in gene expression between antibiotic treated and non-treated macrophages. Relative gene expression was calculated by $\Delta\Delta CT$ method normalised against the CT values of 5 housekeeping genes (Actb, B2m, Gapdh, Gusb and Hsp90ab1) and expressed as fold change (section 2.9.6). Fold-change values >1 indicate increased expression of the genes, while values <1 indicate decreased expression.

Figure 6.1 RNA integrity of ciprofloxacin treated macrophages.
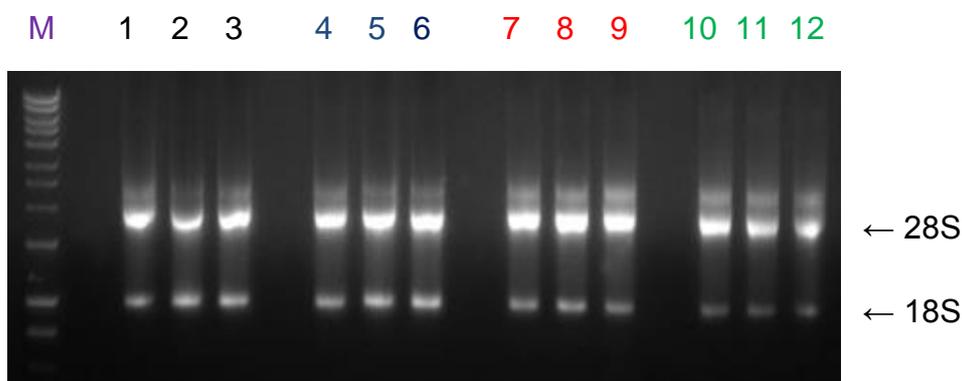
Lane M is 1kb RNA marker. Lanes 1 – 3 (black) contain RNA samples from non-antibiotic treated non-infected macrophages. Lanes 4 – 6 (blue) contains RNA samples from macrophages pre-treated with 5 μg/ml of ciprofloxacin. Lanes 7 – 9 (red) contain RNA samples from macrophages pre-treated with 5 μg/ml of ciprofloxacin and infected with SL1344. Lanes 9 – 12 (green) contain RNA samples from non-antibiotic treated SL1344 infected macrophages.

Figure 6.2 RNA integrity of ceftriaxone treated macrophages.
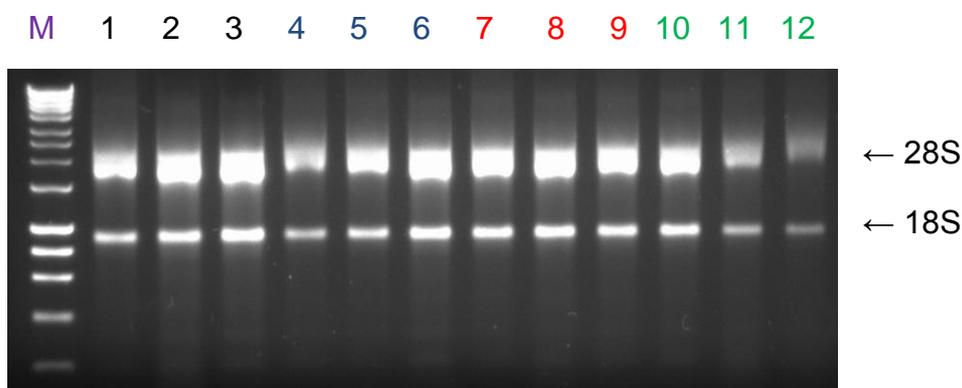
Lane M is 1kb RNA marker. Lanes 1 – 3 (black) contain RNA samples from non-antibiotic treated non-infected macrophages. Lanes 4 – 6 (blue) contains RNA samples from macrophages pre-treated with 82 μg/ml of ceftriaxone. Lanes 7 – 9 (red) contain RNA samples from macrophages pre-treated with 82 μg/ml of ceftriaxone and infected with SL1344. Lanes 9 – 12 (green) contain RNA samples from non-antibiotic treated SL1344 infected macrophages.

A comprehensive list of fold change in gene expression levels of the 84 genes on the RT-PCR array is presented in Appendix 2.

Since the purpose of these experiments was to determine whether antibiotic pre-treatment causes an increased expression of TLR, leading to increased bacterial recognition and binding, the focus was on the genes encoding the expression of *TLR1-9* (Table 6.1 and 6.2) and the inflammatory response to *Salmonella*.

Ciprofloxacin

In comparison to non-antibiotic treated macrophages, in macrophages pre-exposed to 5 µg/ml of ciprofloxacin there was no change in expression of any of the genes of interest (Table 6.1).

<u>*Salmonella* SL1344</u>

In non-antibiotic treated macrophages infected with SL1344, there was increased expression of genes encoding *IL1β, IL6* and *TNFα* by 38, 43 and 21 fold, respectively (p= 0.0001, 0.002 and 0.002 respectively). Expression of *TLR1* and *TLR2* was increased 1.5 and 1.6 fold, respectively, while expression of *TLR 3-9* was decreased (Table 6.1).

<u>*Ciprofloxacin and SL1344*</u>

However when macrophages were pre-treated with ciprofloxacin (5µg/ml) and infected with *Salmonella* SL1344, there was increased expression of *IL1β* and *IL6* by 118 and 143 fold respectively (p= 0.0001, 0.00002), while *TNFα* was increased by 6 fold, a reduction of SL1344 challenged macrophages alone and 0.000338). Expression of *TLR1* and *TLR2* was increased 1.4 and 1.6 fold respectively, (p=0.0014 and 0.0106 respectively), while expression of *TLR3–9* was decreased.

Compared to macrophages infected with SL1344, in ciprofloxacin pre-exposed macrophages infected with SL1344, there was a 3-fold increase in expression of both *IL1β* and *IL6* (Table 6.1). Expression of *TNFα* was decreased 3.5 fold. *TLR 3, 4* and *5* expression was decreased in both ciprofloxacin treated and untreated SL1344

infected macrophages. TLR 8 expression was decreased 2.3 fold in ciprofloxacin and SL1344 treated macrophages compared to non-antibiotic treated macrophages infected with SL1344.

Ceftriaxone

Pre-treatment of macrophages with 82 µg/ml of ceftriaxone, increased the expression of *TLR6* 2 fold (*p*= 0.0369), compared to untreated macrophages. In non-antibiotic treated macrophages infected with SL1344, expression of genes encoding *TLR 3, 4, 5* and *8* was reduced, compared to non-treated macrophages (Table 6.2).

Salmonella SL1344

When macrophages were treated with ceftriaxone and infected with *Salmonella*, there was increased *IL1β* expression (127-fold), 3-fold increase in *IL6* expression, and a 32-fold increase in *TNFα* expression compared to untreated macrophages. *TLR2* expression was increased 5.5 fold in *Salmonella* infected macrophages that had been pre-exposed to ceftriaxone compared to non-treated macrophages (Table 6.2).

Ceftriaxone and SL1344

Comparison of data obtained for non-antibiotic pre-treated macrophages infected with SL1344 and ceftriaxone treated macrophages infected with SL1344 revealed an 8 fold increase in *IL1β* and a 4 fold increase in *IL6* expression in ceftriaxone treated macrophages infected with SL1344 (Table 6.2). By comparison, *TNFα* expression was not further increased. *TLR 3, 4, 5* and *8* expression was equally decreased in both ciprofloxacin treated and untreated, SL1344 infected macrophages (Table 6.2). Consistent with the fold change data, clustergram analysis shows low expression of *IL1β* and *IL6* (green squares) in untreated macrophages and in macrophages pre-treated with ciprofloxacin and ceftriaxone. In non-antibiotic treated macrophages

infected with *Salmonella*, there was moderate gene expression (dark green squares). However, there was high expression (red squares) of these genes in ciprofloxacin or ceftriaxone pre-treated macrophages that were infected with *Salmonella* (Figure 6.3 and 6.4).

Comparison of data obtained for the macrophages pre-treated with antibiotics and macrophages pre-treated with antibiotics and infected with *Salmonella* (Table 6.1 and 6.2) revealed that there was greater expression of *IL1β* and *IL6* in the antibiotic pre-treated macrophages infected with *Salmonella*. Therefore, it was inferred that the increased expression of these genes was due to the pre-exposure of the macrophages to antibiotics. In contrast, there was greater reduction of expression of *TNFα* in *Salmonella* infected macrophages pre-treated with antibiotics than in *Salmonella* infected non-antibiotic pre-treated macrophages.

Table 6.1 Fold change in gene expression of J774 macrophages following pre-treatment with ciprofloxacin (CIP) at C_{max} concentrations (5 µg/ml) compared to untreated macrophages

		Fold change (comparing to non-antibiotic treated macrophages)					
		Macrophage + CIP		Macrophage + SL1344		Macrophage + CIP + SL1344	
		Fold change	p-value	Fold change	p-value	Fold change	p-value
C04	Il1b	0.9659	0.746852	38.9600*	0.000120	118.6044*	0.000995
C07	Il6	0.9330	0.946741	43.0059*	0.002224	143.4968*	0.000015
F08	Tlr1	0.9794	0.941391	1.5630*	0.031977	1.4701*	0.001421
F09	Tlr2	0.8409	0.324562	1.6411*	0.045088	1.6328*	0.010690
F10	Tlr3	0.7631	0.156827	0.3643*	0.000065	0.5526*	0.008224
F11	Tlr4	0.8448	0.663734	0.2006*	0.000377	0.1837*	0.000241
F12	Tlr5	0.9908	0.979696	0.1014*	0	0.1009*	0
G01	Tlr6	0.8467	0.598306	0.6297*	0.085823	0.6154*	0.047654
G02	Tlr7	0.9548	0.781408	0.5681*	0.015783	0.4802*	0.003251
G03	Tlr8	0.8706	0.721758	0.0503*	0.000063	0.1165*	0.000135
G04	Tlr9	0.8746	0.455856	0.6850*	0.085542	0.6114*	0.006805
G05	Tnfα	0.9013	0.507896	21.5995*	0.002455	6.9990*	0.000338

The data summarized in the table illustrate fold changes in the expression level of genes in the TLR signaling pathway of J774 macrophages pre-exposed to ciprofloxacin. The p values are calculated based on a Student's t-test of the triplicate 2^ (- Delta Ct) values for each gene in the control group and treatment groups, and p values less than 0.05 are denoted by *. Increased fold changes are printed in red, while decreased fold changes are printed in blue.

Table 6.2 Fold change in gene expression of J774 macrophages following pre-treatment with ceftriaxone (CRO) at C_{max} concentrations (82 µg/ml) compared to untreated macrophages.

		Fold change (comparing to non-antibiotic treated macrophages)					
		Macrophage + CRO		Macrophage + SL1344		Macrophage + CRO + SL1344	
		Fold change	p-value	Fold change	p-value	Fold change	p-value
C04	Il1b	1.0410	0.668593	15.5050*	0.001923	127.1405*	0.000003
C07	Il6	1.0410	0.668593	0.8461	0.692434	3.6846*	0.027510
F08	Tlr1	0.6204	0.076409	1.3110	0.181982	1.2705	0.246214
F09	Tlr2	0.9126	0.283665	6.5604*	0.000007	5.5229*	0.000009
F10	Tlr3	0.9000	0.488461	0.4400*	0.033508	0.4138*	0.009230
F11	Tlr4	1.0032	0.940031	0.4271*	0.003028	0.4894*	0.002215
F12	Tlr5	1.4320*	0.038840	0.2359*	0.002290	0.1835*	0.000007
G01	Tlr6	2.1307*	0.036946	1.7881	0.150742	1.7905	0.139938
G02	Tlr7	0.7061	0.189922	0.8317	0.102753	0.6790*	0.041310
G03	Tlr8	0.8693*	0.023528	0.2061	0	0.2072*	0.000003
G04	Tlr9	0.7293	0.350244	1.5225	0.143896	1.4636	0.054219
G05	Tnfα	0.8534	0.761438	27.4123*	0.002340	32.7072*	0.000197

The data summarized in the table illustrate fold changes in the expression level of genes in the TLR signaling pathway of J774 macrophages pre-exposed to ceftriaxone. The p values are calculated based on a Student's t-test of the triplicate 2^ (- Delta Ct) values for each gene in the control group and treatment groups, and p values less than 0.05 are denoted by *. Increased fold changes are printed in red, while decreased fold changes are printed in blue.

In these experiments, the RT-PCR assays were carried out in two separate groups, one with ciprofloxacin and the one with ceftriaxone, and performed on different days. Data obtained shows that the same genes were either over expressed or under expressed in both sample groups with the exception of *I6* which was not increased in the ceftriaxone treatment group (Table 6.3). This difference is potentially due to sample to sample variations between replicates used in the different experiments, particularly ceftriaxone treatment of macrophages where there is one clearly different response (Figure 6.4).

Table 6.3 Fold change in gene expression of J774 macrophages following infection with SL1344 compared to non-infected macrophages.

		Macrophage + SL1344 (CIP experiment)		Macrophage + SL1344 (CRO experiment)	
		Fold change	*P*-value	Fold change	*P*-value
C04	Il1b	38.9600*	0.000120	15.5050*	0.001923
C07	Il6	43.0059*	0.002224	0.8461	0.692434
F08	Tlr1	1.5630*	0.031977	1.3110	0.181982
F09	Tlr2	1.6411*	0.045088	6.5604*	0.000007
F10	Tlr3	0.3643*	0.000065	0.4400*	0.033508
F11	Tlr4	0.2006*	0.000377	0.4271*	0.003028
F12	Tlr5	0.1014*	0	0.2359*	0.002290
G01	Tlr6	0.6297*	0.085823	1.7881	0.150742
G02	Tlr7	0.5681*	0.015783	0.8317	0.102753
G03	Tlr8	0.0503*	0.000063	0.2061	0
G04	Tlr9	0.6850*	0.085542	1.5225	0.143896
G05	Tnfα	21.5995*	0.002455	27.4123*	0.002340

Bonferroni correction assessed by dividing a significance level (0.05) by the number of genes tested (84) gave 0.05/84 = 0.000595 which is approximately 0.0006, so any p-values you have below 0.0006 are evidence of a difference at the 5% significance level, in which case most aresults would lose significance. The process assumes that the genes being tested are independent, which they are probably not, hence it is very likely to be conservative. For fold change of genes of interest, further validation by qPCR is required.

Figure 6.3 Clustergram of expression of genes in ciprofloxacin (5 µg/ml) pre-treated J774 macrophages.
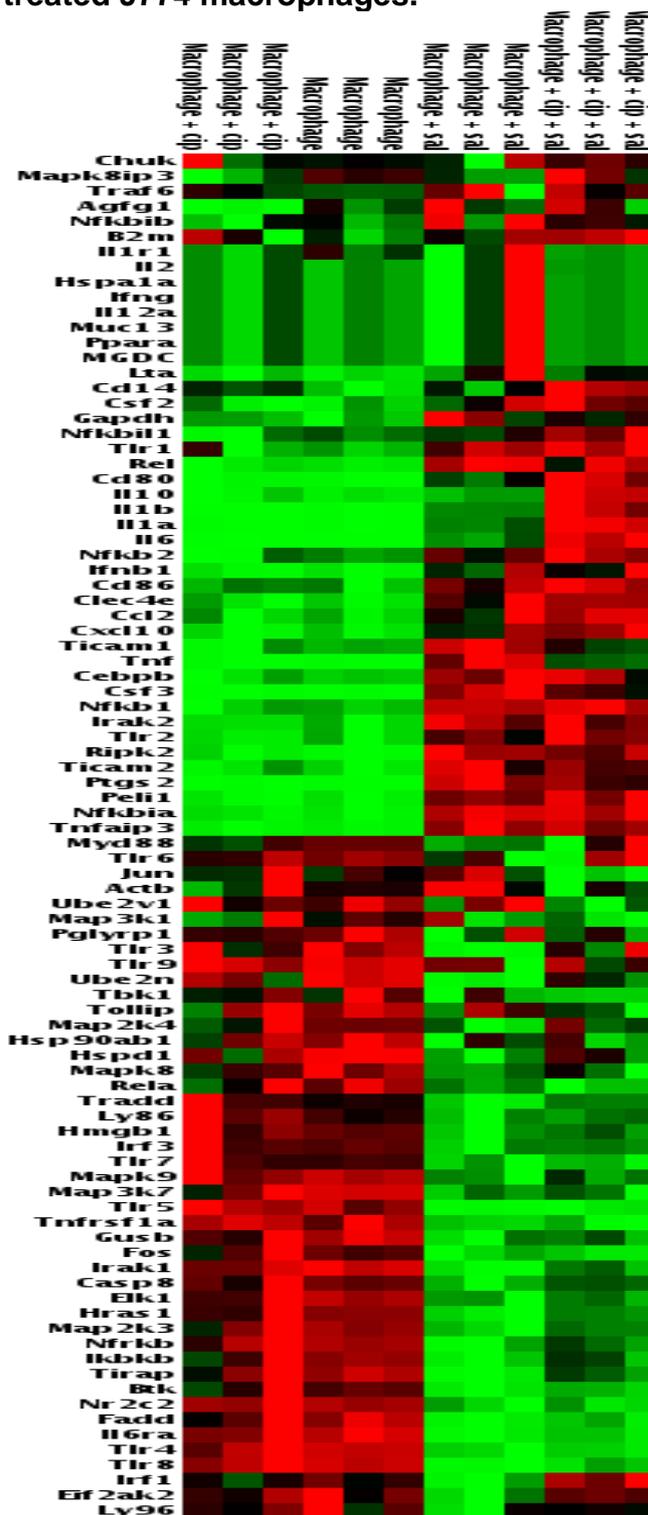
Cluster gram showing the effect of ciprofloxacin on expression levels of genes in the TLR signaling pathway of J774 macrophages. Data are representative of three replicates for each treatment group. Data were median centered using RT² Profiler™ PCR Array Data Analysis software by SABiosciences. Green squares indicate low expression of genes (fold change <1), while red squares indicate high expression of genes (fold change >1).

Figure 6.4 Clustergram of expression of genes in ceftriaxone (82 µg/ml) pre-treated J774 macrophages.
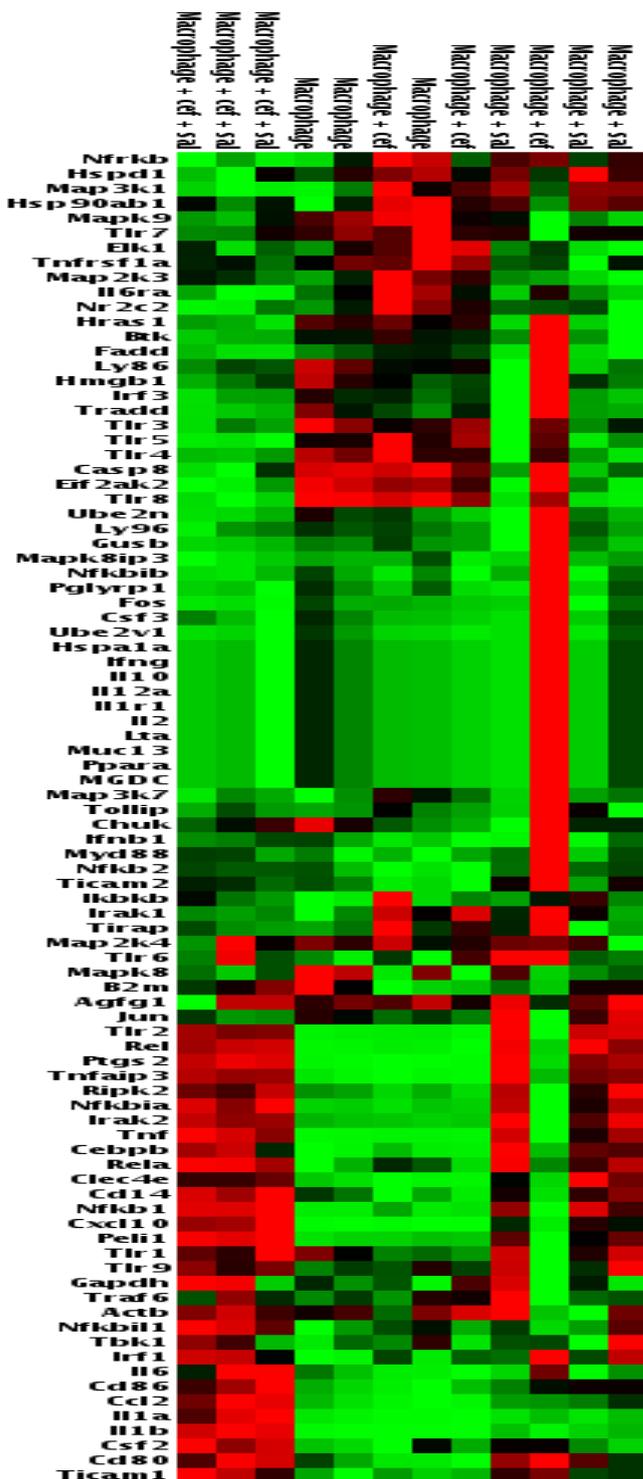
Cluster gram showing the effect of ceftriaxone on expression levels of genes in the TLR signaling pathway of J774 macrophages. Data are representative of three replicates for each treatment group. Data were median centered using RT² Profiler™ PCR Array Data Analysis software by SABiosciences. Green squares indicate low expression of genes (fold change <1), while red squares indicate high expression of genes (fold change >1).

6.4.3 Analysis of IL-1β and TNFα cytokine protein expression in J774 macrophages following antibiotic pre-treatment.

To confirm whether gene expression changes of *IL1β,* and *TNFα* translated to altered protein expression, ELISA analysis using tissue culture supernatant obtained from macrophages in tissue culture experiments (Section 6.4.2) was carried out.

IL-1β protein production

Production of IL-1β was higher in macrophages infected with SL1344 (121 pg/ml) than in non-antibiotic treated, non-infected macrophages (13 pg/ml). Production increased further in ciprofloxacin pre-treated macrophages infected with SL1344 361 pg/ml (Table 6.4 and Figure 6.5). Conversely, IL-1β production was lower in ceftriaxone pre-treated macrophages infected with SL1344, (3 pg/ml) than in ceftriaxone pre-treated, non-SL1344 infected macrophages, 50 pg/ml (Table 6.4 and Figure 6.5).

TNFα protein production

Compared to untreated macrophages, there was higher expression of TNFα in non-antibiotic treated macrophages infected with SL1344 (872 pg/ml) than in non-antibiotic treated, non-infected macrophages (25 pg/ml). Similarly, in ciprofloxacin and ceftriaxone pre-treated macrophages infected with SL1344, TNFα protein expression was higher compared to antibiotic treated non-infected macrophages (Table 6.4, Figure 6.6). Similar to gene expression data, compared to non-antibiotic treated macrophages there was no difference in the level of TNFα produced in macrophages infected with SL1344 (Figure 6.6).

When compared with fold change data in the level of *IL1β* mRNA for antibiotic treated or untreated macrophages, the fold change in protein expression was not consistent.

183

However, the fold change of TNFα mRNA expression in non-antibiotic treated macrophages infected with SL1344 was similar to the fold change in protein expression for this protein compared to non-antibiotic treated, non-infected macrophages (24.5 fold for mRNA vs. 34 fold for protein expression) (Table 6.5).

Table 6.4 Expression of IL-1β and TNFα protein in ciprofloxacin and ceftriaxone treated J774 macrophages.

	Protein (pg/ml)	
Sample	IL-1β	TNFα
Macrophage only	13.3995	25.6062
Macrophage + SL1344	120.9990	871.8641
Macrophage + CIP	32.3380	71.7853
Macrophage + CRO	50.7265	31.0034
Macrophage + CIP + SL1344	361.9135	705.6677
Macrophage + CRO + SL1344	2.9540	1002.1006

Table 6.5 Comparison of fold change in mRNA and protein expression in ciprofloxacin and ceftriaxone treated J774 macrophages.

Fold change compared to non-antibiotic treated macrophages				
	IL-1β		TNFα	
Sample	mRNA	Protein	mRNA	Protein
Macrophage + SL1344	27.2325*	9.0301	24.5059*	34.0489
Macrophage + CIP	0.9659	2.4134	0.9013	2.8034
Macrophage + CRO	1.0410	3.7857	0.8534	1.2108
Macrophage + CIP + SL1344	118.6044	27.0095	6.9990	27.5584
Macrophage + CRO + SL1344	127.1405	0.2205	32.7072	39.1350

* Macrophage + SL1344 mRNA results are an average of fold change data from samples from both days of the experiment

Figure 6.5 IL-1β protein production in J774 macrophages pre-treated with ciprofloxacin (5 µg/ml) or ceftriaxone (82 µg/ml).
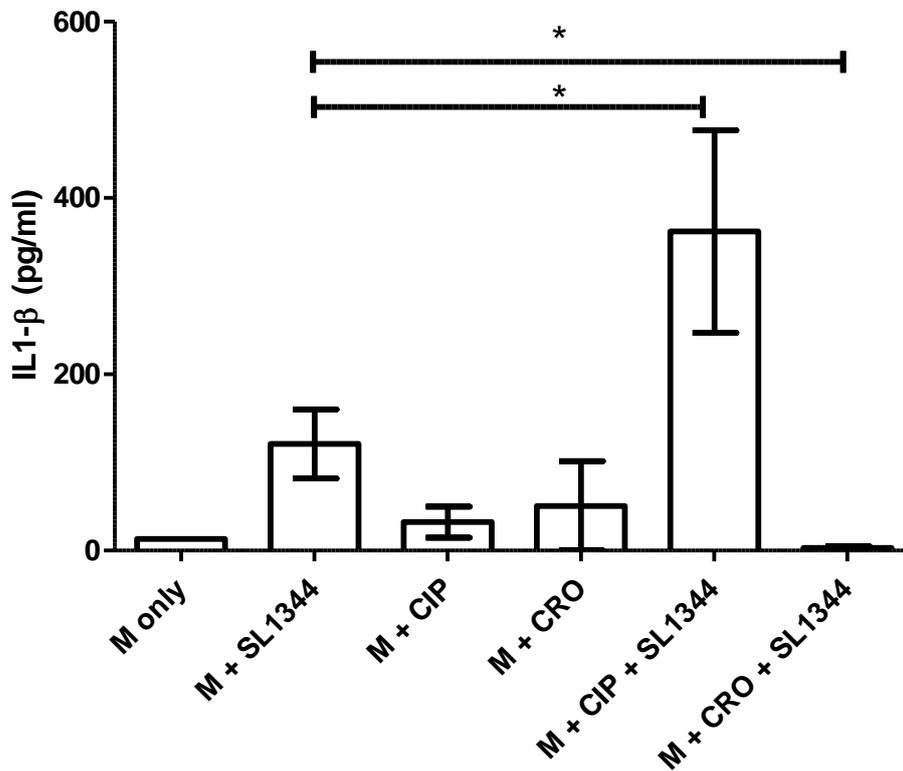
Capture ELISA analysis of IL-1β in response to SL1344 in J774 macrophages pre-treated with ciprofloxacin or ceftriaxone for 2 hours. Data represents means of three experiments performed in triplicate. Standard error represents error between 3 biological replicates and 3 technical replicates. A student's T test was carried out to compare cytokine expression in antibiotic pre-treated and non-treated macrophages challenged with *Salmonella*. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

Figure 6.6 TNFα protein production in J774 macrophages pre-treated with ciprofloxacin (5 µg/ml) or ceftriaxone (82 µg/ml).

Capture ELISA analysis of TNFα in response to SL1344 in J774 macrophages pre-treated with ciprofloxacin or ceftriaxone for 2 hours. Data represents means of three experiments performed in triplicate. Standard error represents error between 3 biological replicates and 3 technical replicates. A student's T test was carried out to compare cytokine expression in antibiotic pre-treated and non-treated macrophages challenged with *Salmonella*. The * indicates a significant difference ($p<0.05$), all other comparisons were considered non-significant, n=3.

6.5   Discussion

The hypothesis investigated in the experiments described in this chapter was that under antibiotic, pre-treatment of macrophages leads to increased expression of TLR leading to increased microbial binding of *Salmonella* by TLR, hence explaining the increased adhesion in the tissue culture model of *Salmonella* infection. There is currently no available literature that elucidates how antibiotics affect TLR signaling in response to *Salmonella*. Therefore, cellular and molecular mechanisms for the effect of antibiotics on TLR are still poorly understood. My data showed decreased fold-change expression of TLR in *Salmonella* infected antibiotic treated, or untreated, J774 macrophages. However, activation of TLR in response to *Salmonella* is characterized by increased production of cytokines such as IL-1β, IFN-γ and IL-6 (Huang, 2009, Svensson et al., 2001, Sebastiani et al., 2002, Cronin et al., 2012). In agreement with these authors, my data also showed increased expression of *IL1β*, *IL6* and *TNFα* when macrophages were infected with *Salmonella*. My data further reveals that ciprofloxacin pre-treatment caused greater expression of *IL1β* and *IL6* than in non-ciprofloxacin treated macrophages. Similarly, ceftriaxone pre-treatment increased the expression of *IL1β* by 112 fold, and slightly increased the expression of *IL6*. Additionally, my data showed that both ciprofloxacin was able to reduce expression of *TNFα* in pre-treated macrophages infected with *Salmonella*. These findings support those obtained by Gogos et al., (2004) who showed that ciprofloxacin inhibited the production of TNFα, but not IL-6, in the sera of patients suffering from sepsis (Gogos et al., 2004). Although not at gene level, it has been shown that ciprofloxacin inhibited the production of TNFα, IL-1β and IL-6 in LPS stimulated mouse peritoneal macrophages pre-treated with ciprofloxacin for 1 hour

(Ogino et al., 2009). These authors suggest that the ability of ciprofloxacin and other fluoroquinolone antibiotics to modulate the immune response is due to the presence of cyclopropyl group at the N1 position or piperazinyl at the C7 position of a tricyclic benzoxazine nucleus (Ogino et al., 2009).

A limitation of the current study is that certain experiments gave contradictory results, such as ceftriaxone treatment of macrophages alone. Therefore these results must be considered preliminary until further studies are performed to confirm the effects of antibiotics on the TLR pathway at the gene, but also at the protein level, where the kinetics of the response may differ.

TLR are important for induction of the innate immune response and cytokine expression. Consistent with reduced induction of innate immune response, it has been shown that deficiency in *TLR2* and *TLR4* in mice is correlated to increased susceptibility to *S.* Typhimurium infection (Arpaia et al., 2011).

Despite being important effector mechanisms for recognition of pathogens, TLR are exploited by pathogens in establishing and maintaining infection in the host. TLR signaling induces acidification of the *Salmonella*-containing phagosome (Arpaia et al., 2011). In this acidified environment, there is increased expression of *Salmonella* type three secretion systems (TTSS) encoded by SPI-2, whereas genes encoding the secretion of TTSS by SPI-2 are disrupted in a non-acidic environment (Cirillo et al., 1998). Since SPI-2 is required for replication of and systemic infection by *Salmonella* in the host and TLR signaling facilitates the expression of SPI-2, TLR signaling is beneficial as well as detrimental to the host. Hence identification of antibiotics that are able to modulate TLR signaling is vital in treating infections where TLR signaling is a key immune response.

Previous reports have stated that in response to *Salmonella* infection, TLR2, TLR4 and TLR5 signaling are required (Seibert et al., 2010). However, activation of these receptors occurs at different points in the immune system's encounter with *Salmonella*, i.e. TLR responses to bacteria are sequentially activated. For instance, Weiss et al., (2004) showed that although TLR4 is required for initial cytokine production and killing of *Salmonella*, it is also required for the downstream response such as induction of myeloid differentiation factor 88 (MyD88), a TLR adaptor protein involved in the activation of transcription factor NF-κB (Weiss et al., 2004). Data from my experiments showed that after 2 hours, infection of antibiotic pre-treated or untreated macrophages with *Salmonella* failed to up regulate the expression of TLR encoding genes. As these genes are transiently expressed, it is possible that these genes had been "switched on" and later "switched off" in course of the 2 hour infection period, and so affecting the data obtained.

In the presence of *Salmonella*, ciprofloxacin inhibited the expression of *TLR4*. Further, reduced expression of *TNFα* was seen in *Salmonella* infected ciprofloxacin-treated macrophages. A similar result was obtained in work by Katsumo et al., (2006) using human monocytes, where at concentrations ranging from 0.1 to 100 µg/ml, ciprofloxacin suppressed the expression of *TLR4* and the production of TNFα. Further, they reported that ciprofloxacin at the same concentration range also inhibited the expression of intracellular adhesion molecules ICAM-1, B7.1, B7.2 and CD14 (Katsuno et al., 2006). ICAM-1 is involved in cell to cell signaling and transmigration into tissues (Grasso et al., 2014). These authors hypothesized that inhibition of interaction between monocytes via reduction in the expression of ICAM and CD14 by ciprofloxacin may be responsible for the reduced expression of TNFα in monocytes.

In the THP-1 human macrophage cell line, Bode et al., (2014) also illustrated the differential effects of antibiotics on TLR gene expression, cytokine production and phagocytosis after 24 hour incubation. Their data showed increased expression of *TLR1, TLR2, TLR4*, *TLR6*, *IL1β* and *IL6* genes when macrophages were pre-treated with the beta-lactam antibiotic piperacillin (100µg/ml), and stimulated with LPS from *E. coli*. However, pre-treatment with the tetracycline antibiotic, doxycycline (8ug/ml) or the aminoglycoside, gentamicin (5 µg/ml) reduced mRNA expression of *TLR1, TLR4, TLR6*, and *IL1β*. In peripheral blood mononuclear cells from patients with a systemic inflammatory response who had undergone surgery, differential effects of antibiotics in modulating expression of TLR and cytokine genes were observed (Bode et al., 2014). More specifically in the presence of LPS, the fluoroquinolone antibiotic moxifloxacin increased mRNA levels of *TLR2*, but decreased expression levels of *TLR1*. However, in the absence of LPS, expression of *IL1β* was increased while expression of *TLR4* and *TLR6* was decreased. Other antibiotics have also been recently shown to interfere with expression of immune response genes. For instance, erythromycin and rifampicin prevented the over expression of *TLR4, TLR2, CD18* and *IL8r* in response to LPS, and also suppressed the expression of *TNFα* genes in human polymorphonuclear leukocytes (Mu et al., 2014). However, the mechanisms underlying these findings have not been elucidated.

It is has been shown that TLR signaling is required for the production of pro-IL-1β, which is cleaved into IL-1β (Martinon et al., 2002, Dinarello, 2007). Although my data shows that expression of *TLR* genes was not increased in response to SL1344 or antibiotics in J774 macrophages, it is hypothesised that TLR are constitutively expressed in these cells and are up regulated in response to *Salmonella* and then switched off thereafter. Hence, measuring expression of these genes 2 hours after

infection with *Salmonella* may not give a true representation of expression of these genes. Foster et al., (2007) suggested that *TLR* can be transiently expressed, after which they induce the expression of pro-inflammatory mediators and priming of antimicrobial functions (Foster et al., 2007). Although infection with of macrophages with *Salmonella* for 2 hours is suitable for measuring invasion and adhesion (Dibb-Fuller et al., 1999), there are some caveats to the use of this method for measuring gene expression. For instance, it may be possible that transient expression of TLR genes occurred when macrophages were infected with SL1344. Therefore, further work should include monitoring of the expression of these TLR genes upon infection with *Salmonella* and at shorter time points after infection.

The effect of antibiotics in modulating the immune response, especially pathogen recognition and cytokine production has various clinical implications. Some reports have highlighted TLR as new therapeutic targets for the treatment of infections (Roger et al., 2009, Savva and Roger, 2013). Furthermore, several classes of antibiotics, currently used in treatment against bacterial infections modulate the immune response, especially the expression of TLR and pro-inflammatory cytokines (Bode et al., 2014). These immunomodulatory effects vary between antibiotics of various classes. Antibiotics belonging to the same class can also affect the immune response in different ways. For instance cefuroxime and ampicillin, both beta-lactam antibiotics have opposing effects on immune related gene expression in human T cells. Cefuroxime increased the expression of genes involved in Th2 and Treg pathways, ampicillin increased the expression of genes in the TH1 pathway (Mor and Cohen, 2012), while macrolide immunomodulatory mechanisms are mainly in the regulation of inflammation and neutrophil activities (Shinkai et al., 2008). These

findings suggest that antibiotics differentially affect the immune response, and these effects vary in the presence or absence of bacteria.

In summary, my work shows that ciprofloxacin and ceftriaxone, at the concentration and time of exposure to antibiotics replicating the C_{max} and T_{max} respectively, influence *TLR* mRNA expression and cytokine response to SL1344 at both gene and protein level in J774 macrophages. My data showed that the antibiotics, ciprofloxacin and ceftriaxone further increased the expression of genes that were previously over expressed when macrophages were infected with *S.* Typhimurium SL1344, but did not alter genes with repressed expression following infection with SL1344. These findings indicate that antibiotics have higher ability to influence the immune response in the presence of an infection but less so in the absence of an infection. Further, the ability of antibiotics to modulate expression of cytokines may be useful in minimising tissue damage during infection. Increased TNFα production is a 'double-edged sword' as it is involved in increased migration of immune cells from the blood and activation of macrophages to fight infection (Behnsen et al., 2015). However, in severe infections such as during sepsis, there is an increase in TNFα which causes tissue damage while, on the other hand, it is proangiogenic *in vivo.* During the inflammatory process, TNFα can inhibit its own activities by producing soluble receptors. TNFα will also drive production of IL-10 in neutrophils. IL-10 keeps inflammation under control by selectively inhibiting the cell recruitment to sites of infection. IL-1β also increases expression of adhesion factors on endothelial cells to enable transmigration into tissues. Moreover, IL-1β affects the activity of the hypothalamus, which leads to a rise in body temperature, one of the cardinal signs of inflammation, vasodilation and hypotension. It should be stated that the differences

seen in cytokine production with different antibiotics would need to be confirmed at different (optimal) time points for each drug.

6.6   Key points:

- Pre-exposure to antibiotics altered mRNA levels of genes in the TLR signaling pathway of J774 macrophages.

- Pre-treatment of macrophages with ciprofloxacin and ceftriaxone alone did not affect cytokine production or TLR signaling in J774 macrophages.

- Expression of IL-1β and TNFα mRNA was greater in SL1344 infected macrophages which had been pre-exposed to ciprofloxacin or ceftriaxone, than in macrophages exposed to antibiotics alone or SL1344 alone.

- mRNA expression for TLR 3, 4, 5, and 8 was decreased in both non-antibiotic treated macrophages infected with SL1344 and in ciprofloxacin or ceftriaxone pre-treated, SL1344 infected macrophages.

- Production of IL-1b cytokine in ciprofloxacin and ceftriaxone pre-treated macrophages in response to SL1344 was not consistent with mRNA expression, where the fold change in production of this cytokine was lower than the fold change in its mRNA expression.

- Compared to non-antibiotic treated macrophages infected with SL1344, mRNA levels of TNFα were decreased in ciprofloxacin treated macrophages infected with SL1344 but, increased in ceftriaxone treated macrophages infected with SL1344. However, this did not correlate with protein expression of this cytokine in ceftriaxone treated macrophages.

6.7   Future work:

- Based on data obtained in chapter 4 where there was increased adhesion to antibiotic treated macrophages, it was hypothesised that increased adhesion of *Salmonella* to macrophages in *in vitro* tissue culture experiments was due to increased expression of TLR in the macrophages. However, mRNA expression 2 hours after incubation with *Salmonella* did not correlate with this hypothesis. Some antibiotics such as azithromycin show time dependent effects on immune cells because they persist within the body for weeks. Circulating levels of such antibiotics can initially enhance immune functions immediately after administration of the drug (Culic et al., 2002), and can reduce inflammatory responses days after administration (Parnham et al., 2014). Therefore, gene expression of the TLR genes at short time intervals e.g. at the time of *Salmonella* infection and every 10 minutes after for a longer time course (beyond 2 hours) should be determined.

- Since IL-1β is highly expressed by antibiotic treated macrophages, activation of the inflammasome in response to *Salmonella*, in the antibiotic treated or untreated macrophages should be determined.

- Using a protein transport inhibitor (e.g. Brefeldin A), investigate intracellular production of cytokines within macrophages in response to antibiotic pre-treatment and SL1344 infection.

CHAPTER SEVEN

7 Overall discussion and conclusion

7.1 Discussion

The emergence of antibiotic resistance has become a serious problem as infections are becoming more difficult to treat, and so alternative approaches for the treatment of bacterial infections are being sought. There is increasing evidence to suggest that the immunomodulatory properties of some antibiotics are beneficial for the treatment of infections, and studies are beginning to examine the combined action of antibacterial agents and the immune response in clearing infections. As reviewed by Nathan et al., antibacterial therapy targeting infections at the host level must fulfil one of two requirements i) enhancing the host's immunity, or ii) blocking aspects of the host immune system that the pathogens exploit to cause disease (Nathan, 2012).

The aim of this study was to investigate the effect of antibiotic therapy upon the innate immune response to bacteria. This was achieved by exploring the effect of antibacterial agents on i) bacteria in the absence of immune cells, ii) neutrophil functions such as phagocytosis, oxidative burst and killing and iii) on the interaction between macrophages and *Salmonella* by measuring bacterial adhesion and invasion, and expression of genes in the TLR signalling pathway.

I investigated the effect of antibiotics on the growth of three isogenic strains of *Salmonella enterica* serovar Typhimurium wild type SL1344 (L354), SL1344 *tolC::aph* (L109) and SL1344 Δ*aroA* (L730). At concentrations mimicking the maximum human serum levels of the drug, and in the absence of immune cells, two antibiotics ciprofloxacin and ceftriaxone reduced the viable counts of all three strains, 30 and 60 minutes post antibiotic exposure. The SL1344 *tolC::aph* and SL1344 Δ*aroA* stains were more susceptible to these antibiotics than wild type SL1344. C_{max}

concentrations of azithromycin, tetracycline and streptomycin had no effect on the viability of bacteria. At MIC concentrations of the ceftriaxone, SL1344 *tolC::aph* and SL1344 Δ*aroA,* but not wild type SL1344, were susceptible to antibiotic exposure. Ciprofloxacin, tetracycline, azithromycin and streptomycin at MIC concentrations did not alter viability of any of the three strains. Next, using a neutrophil killing assay, I further investigated if the presence of neutrophils enhances the efficacy of bacterial killing by these antibiotics. Neutrophil experiments were carried out using previously described protocols with slight modifications (Cacchillo and Walters, 2002, Iskandar and Walters, 2010). The results of these experiments showed that at bacteria to neutrophil ratios of 100:1 (for all three strains of bacteria), pre-exposure of neutrophils to the C_{max} concentration of ceftriaxone enhanced the killing of *S.* Typhimurium, while pre-exposure to ciprofloxacin, tetracycline, azithromycin or streptomycin did not alter killing of *S.* Typhimurium by neutrophils. It has previously been demonstrated that at bacteria to neutrophil ratios of 30:1, it is difficult for neutrophils to clear bacterial infections (Cacchillo and Walters, 2002). Hence, the increased efficiency of neutrophils to kill bacteria at an even higher bacteria to neutrophil ratio of 100:1 observed in this study is attributed to the effect of ceftriaxone on the neutrophil making them more efficient at killing bacteria.

It has been speculated that the ability of some antibiotics to accumulate inside neutrophils supplements the capacity of the neutrophils to kill bacteria (Cacchillo and Walters, 2002). Although ceftriaxone accumulates poorly in neutrophils (Gemmell, 1993), exposure to this antibiotic enhanced the ability of neutrophils to kill *S.* Typhimurium compared to neutrophils that had not been exposed to the drug. In contrast, my findings showed that ciprofloxacin, which efficiently accumulates within

neutrophils and increases their antibacterial activity (Girish et al., 2013), did not alter neutrophil ability to kill *Salmonella*. This finding is supported by previous authors who posit that intracellular accumulation alone does not increase an antibiotic's propensity to enhance the immune response (Hand and King-Thompson, 1986, Lin and Lu, 1997). Other beta-lactam antibiotics such as cefuroxime, penicillin and ampicillin have also been shown to alter the activities of immune cells such as T cells (Mor and Cohen, 2012). Genes involved in T helper 2 (Th2) and T regulatory (Treg) differentiation were down-regulated in T cells treated with cefuroxime. It was suggested that these immune modulatory activities of the beta-lactam antibiotic were due to covalent binding to cellular albumin (Mor and Cohen, 2012). This observation may give some insight into the ability of ceftriaxone to alter neutrophil activities as reported in my study, despite its inefficient accumulation within these cells. If ceftriaxone binds to neutrophil cellular albumin, it may be possible that when in contact with the neutrophils, bacteria are exposed to albumin bound drug, hence the increased killing found in ceftriaxone treated neutrophils. As suggested by Mor and Cohen (2012), binding of ceftriaxone to albumin greatly extends the half-life of this antibiotic and therefore compared to unbound antibiotic, it will have a longer time to activate neutrophils. This may in turn prolong any antibiotic induced effect such as increased bacterial killing by neutrophils. Albumin is a blood protein produced by the liver and is involved in maintaining osmotic pressure in the vascular system but also serves as a carrier for a variety of blood molecules and drugs (Evans, 2002). Since there is limited literature on albumin production by immune cells, further investigation to determine whether albumin is produced by neutrophils, and to investigate whether ceftriaxone for instance, binds to the albumin should be carried out in order to evaluate its implications for the modulation of neutrophil response to bacteria. It is

also suggested that the protein binding capacity of antibiotics increases the potential of such drugs to reach infected compartments, especially for infections localised in compartments with low immune defences such as the CSF (Van Bambeke and Tulkens, 2009).

I also investigated phagocytosis and oxidative burst in antibiotic treated or untreated neutrophils to determine whether these processes were enhanced in the presence of antibiotics. My results show that neutrophil phagocytosis in response to FITC labelled opsonised *E. coli* was impaired in ciprofloxacin pre-treated neutrophils. It has been established that ciprofloxacin accumulates within neutrophils and other phagocytic cells (Rispal et al., 1996, Lemaire et al., 2011, Michot et al., 2005). However, following accumulation, the effect of this antibiotic on the cellular functions of these eukaryotic cells has not been defined. There were no observed changes in phagocytosis for neutrophils pre-exposed to tetracycline, streptomycin or azithromycin. Moreover, neutrophil superoxide production in response to the opsonised *E. coli* was significantly higher in ceftriaxone pre-treated neutrophils than in untreated cells. Some of the results of previous studies describing the effect of antibiotics on immune response to bacterial challenge are inconsistent and conflicting. While some studies showed that ciprofloxacin enhanced killing of *S. aureus* by pre-treated neutrophils but did not affect phagocytosis (Forsgren and Bellahsene, 1985), other authors showed that ciprofloxacin failed to enhance killing of *S. epidermidis* (Pascual et al., 1989). However, my data showed that phagocytosis and killing of *Salmonella* was impaired in neutrophils pre-treated with ciprofloxacin. Perhaps, the use of different bacterial strains, immune cells from different animals or mice strains, the type of antibiotics, and the technique used contributes to the

inconsistencies associated with investigating antibiotic effects on host responses to bacterial infection. Labro et al., (1987) further showed that the effect of ceftriaxone on neutrophil phagocytosis and killing varied between neutrophils in suspension and adherent neutrophils, with ceftriaxone decreasing bacterial phagocytosis and killing in adherent neutrophils but having no effect on neutrophils in suspension.

One caveat to the experiments for exploring the killing of bacteria by antibiotic treated and untreated neutrophils is the maintenance of the antibiotics in the medium. Since these antibiotics are capable of inhibiting bacterial growth, this makes it difficult to distinguish between the effects of the antibiotics on the neutrophils vs. the effects on the bacteria (Root et al., 1981). However, the rationale behind my experimental design was to mimic the *in vivo* situation where the antibiotic is in contact with the immune system and bacteria are also exposed to concentrations of the drug in serum or in tissue. Cacchillo and Walters also explained that within *in vitro* experimental protocols antibiotics are maintained in the culture medium, as removal of the antibiotic promotes efflux of the agent (Cacchillo and Walters, 2002).

Since macrophages are central to the systemic spread of *Salmonella* within the host, I also investigated the interaction between *Salmonella* and macrophages pre-exposed to antibiotics. Gog et al., lists four key mechanisms that are essential for *Salmonella* to establish an infection in the host, these are: adhesion, invasion, survival and proliferation. This study examined two of the four mechanisms; adhesion and invasion (Gog et al., 2012).

The tissue culture model of *Salmonella* infection was chosen because this is an established model that allows investigation and understanding into the molecular mechanisms of *Salmonella* infection and host responses to infection (Hurley and

McCormick, 2003). This model has been previously used in the Piddock Laboratory where it was shown that the MOI (100:1) and the time scale relevant for the experiment does not cause any toxicity to the macrophage cells (Buckley et al., 2006, Blair et al., 2009). In order to measure the effect of antibiotics using this model, adherent macrophage cells (J774s, mouse bone marrow derived macrophages, THP-1 and human monocyte derived macrophages) were pre-exposed to C_{max} concentrations of the various antibiotics for 2 hours prior to infection of the cells with *Salmonella.* To the best of my knowledge, there is no previous literature describing adhesion to, or invasion of, *Salmonella* to antibiotic pre-treated macrophages. Overall, my data showed that bacterial adhesion to antibiotic pre-treated J774 macrophages was significantly higher than in non-treated macrophages. Comparison of adhesion and invasion data for non-antibiotic treated macrophages with previously published work by Blair et al., (2009) showed similar levels of adhesion of SL1344, but not invasion, with the results in this study. Adhesion of wild type SL1344 to macrophages pre-treated with ciprofloxacin, ceftriaxone, azithromycin and streptomycin was enhanced, but not when macrophages were treated with tetracycline. Invasion of SL1344 was only increased by tetracycline. It has previously been established that inactivation of *tolC* reduced the ability of *Salmonella* to adhere to, or invade host cells (Blair et al., 2009). The SL1344 SPI-1::*aph* strain was used because it is a less virulent and invasive strain, with an impaired SPI locus responsible for translocation of virulence proteins in the invasion process. Hence, any significant changes in the adhesion to, or invasion of, macrophages by these two strains can be attributed to pre-exposure of the macrophages to antibiotics. The SL1344 ∆*aroA* strain was used as a control because although it is a less virulent strain, it does not have any impairment in adhesion and invasion of host cells.

Therefore, any changes in the levels of adhesion or invasion can be attributed to antibiotic effect on the macrophages. Adhesion of SL1344 *tolC::aph* did not vary between antibiotic pre-treated and non-treated macrophages, but surprisingly invasion of antibiotic treated macrophages was generally increased. Antibiotic pre-treatment of macrophages had varying effects on the invasion and adhesion of SL1344 Δ*aroA* and SL1344 SPI-1::*aph*. Generally, this showed that when macrophages have been pre-exposed to antibiotics, adhesion of wild type SL1344 is enhanced but not the other isogenic strains SL1344 *tolC::aph*, SL1344 Δ*aroA*, and SL1344 SPI-1::*aph* while invasion was enhanced for the less virulent *tolC* mutant strain. This observation poses questions; i) is increased adhesion of *Salmonella* to macrophages advantageous or detrimental to the progression of *Salmonella* infection? ii) are there mechanisms within the macrophages that are altered when exposed to antibiotics which makes a less virulent bacterium more able to invade or persist in the cell? Increase in bacterial adhesion to immune cells such macrophages could suggest that there will be increased phagocytic activity of the macrophages, and the infection cleared faster. Uncontrolled phagocytic activities on the other hand might elicit inflammatory responses which are detrimental to host tissues. Future studies investigating how bacterial adhesion to immune cells such as macrophages can be modulated by antibiotics should be considered.

I hypothesised that the antibiotics, ciprofloxacin and ceftriaxone altered expression of TLR on macrophages, increasing binding of *Salmonella* to TLRs hence the increased adhesion of *Salmonella*. Therefore, to investigate the mechanism behind the increased adhesion of bacteria to antibiotic exposed macrophages and its implications for the progression of *Salmonella* infection, expression of genes in the

TLR signalling pathways of J774 macrophages in response to *Salmonella* was investigated using quantitative RT-PCR assay on mRNA from macrophages. The major TLR association for *Salmonella* is TLR5 through binding of flagellin, while TLR4 recognises *Salmonella* LPS (Arpaia et al., 2011). The gene expression data showed higher expression of genes encoding TLR2 in macrophages treated with ceftriaxone. Although further investigation is required to validate these data, they suggest a link between expression of TLR2 genes under ceftriaxone exposure and adhesion of *Salmonella*. This could include several TLR and potentially other pattern recognition receptors (Arpaia et al., 2011, O'Donnell and McSorley, 2014). The cytokine genes IL1β and IL6 were more highly expressed in antibiotic pre-treated macrophages compared to untreated macrophages following *Salmonella* infection. In contrast, TNFα gene expression was repressed in antibiotic pre-exposed macrophages. IL1β and IL-6 are important in bacterial infections as they activate the recruitment of neutrophils to sites of infection, induce the activation of endothelial adhesion molecules, cytokines and chemokines and stimulate T cell responses (Sahoo et al., 2011, Scheller et al., 2011). Interestingly, findings of the RT-PCR assays showed genes encoding the expression of TLR 1-9 were less expressed after infection with *Salmonella*. This can be explained by the findings of O'Mahony et al., (2008) and Juarez et al., (2010) which suggested that TLRs are constitutively expressed; hence the gene expression profile is not altered in the presence or absence of antibiotics (O'Mahony et al., 2008, Juarez et al., 2010). IL-1β is important against bacterial infection as it is an activator of neutrophils and macrophages, leading to the phagocytosis of the invading pathogen and release of oxygen and nitrogen radicals by these cells. Further, IL-1β activates the release of pro-inflammatory cytokines such for instance TNFα and IL-6 (Netea et al., 2010).

203

An important factor in these experiment using J774 macrophages is that these are phagocytic cell lines. Although these macrophages were not pre-activated prior to *Salmonella* infection, the possibility that some of the bacteria may have been phagocytosed was not excluded. Hence, I carried out experiments where macrophages were pre-treated with cytochalasin D at 10 µg/ml hour to inhibit phagocytosis (Bosedasgupta and Pieters, 2014). The data obtained showed no significant difference in bacterial adhesion and invasion between the cytochalasin treated and untreated groups in the presence or absence of antibiotics.

One interesting observation in the tissue culture infection assays was the variations between bacterial adhesion to antibiotic pre-treated murine macrophages and antibiotic pre-treated human macrophages (primary and cell line). Data shown in Chapter 4 revealed that there was increased bacterial adhesion to ciprofloxacin and ceftriaxone treated J774 macrophages and murine BMDMs but not in THP-1s or human MDMs when challenged with *S*. Typhimurium, which causes enteric fever in mice but not in humans. This observation draws attention to the need to reassess the use of mouse models for simulating infections and other disease conditions in humans. The use of mouse models of human diseases has been the object of recent debate. Seok et al., (2013) argue that mouse models poorly correlate with genomic responses in human conditions and should not be relied on for studying human diseases. Their findings were however refuted by Takao and Miyakawa, (2014) who demonstrated that gene expression patterns in mouse models showed significant correlations with those of human conditions, and argued that the failure to detect correlation resulted from inappropriately biased methodologies they used (Seok et al., 2013, Takao and Miyakawa, 2014). Although the mouse immune system is

related to the human immune system, they are not the same. For instance, in mouse macrophages, iNOS and NO expression is induced by TLR and IFN-γ signalling but not in human MDMs (Schneemann and Schoeden, 2007, Mestas and Hughes, 2004). These variations could be due to genetic or environmental factors, which are more controlled in murine cells than in human cells. Translating findings of *in vitro* experiments into the context of an animal model and subsequently to human disease remains a difficult challenge for any disease process. All the gene expression experiments in this study were carried out using murine macrophages. In order for these findings to be clinically validated, it is important to consider the already existing differences between the murine immune system and the human immune system when extrapolating results from mice to humans. It is possible that other factors such as efficiency of expression of receptors can influence the outcome of the infection assay. Previous studies have indicated that efficiency of expression of receptors such as Fcγ and the efficacy of phagocytosis are a function of the macrophage cell cycle (Luo et al., 2006). Therefore, adhesion and invasion, as well as expression of TLR should be investigated at different stages of the cell cycle in the presence or absence of antibiotic treatment.

Altogether, my data showed antibiotic-dependent effects upon immune cell functions (Table 7.1). Tetracycline had no effect on neutrophil functions and did not affect adhesion to, or invasion of, *Salmonella* SL1433 to J774 macrophages. Azithromycin had no effect on the neutrophil response to bacteria but increased *Salmonella* SL1344 adhesion to J774 monolayers at concentrations below the C_{max}, whereas above the C_{max} it decreased invasion. Ciprofloxacin and ceftriaxone showed the most consistent effects in the experiments. Although ceftriaxone had no effect on

neutrophil phagocytosis, there was an increased oxidative burst in neutrophils exposed to this drug. It is possible that this ability of ceftriaxone to enhance neutrophil oxidative burst explains the increased killing of *Salmonella* when incubated with ceftriaxone pre-treated neutrophils (Section 5.5). In contrast, ciprofloxacin reduced neutrophil ability to phagocytose bacteria, but did not affect the oxidative burst. This inhibition of phagocytosis could explain its lack of effect on neutrophil ability to kill *Salmonella*. Streptomycin increased adhesion of *Salmonella* SL1344 to J774 macrophages at C_{max} concentration.

My research is the first to systematically analyse the effect of commonly used antibiotics on the responses of neutrophils and macrophages to *Salmonella* Typhimurium infection. Whilst further studies are now warranted, data presented in this thesis shows that ceftriaxone had the most consistent effect on macrophage and neutrophil interaction with bacteria. Increased bacterial oxidative burst correlated with enhanced bacterial killing in ceftriaxone treated neutrophils, while enhanced expression of gene encoding TLR2 could be associated with increased bacterial adhesion to ceftriaxone treated macrophages.

Based on the findings of this thesis, the use of ceftriaxone is recommended for treatment of *Salmonella* infections as it has shown consistent effects not only in reducing bacteria viability but also in enhancing neutrophil and macrophage response to *Salmonella* infection. This is also further supported by super array data showing increased expression of TLR 2 in ceftriaxone treated macrophages in response to *Salmonella* infection. However, resistance to ceftriaxone due to beta-lactamases poses a challenge to ceftriaxone administration. This raises the need for development of new antibiotics with potent anti-beta lactamase activity.

7.2 Conclusion

In conclusion, using different models, my data show that administration of antibiotics can modulate the immune response to bacteria, in addition to their antibacterial properties. Antibiotic therapy is the basis of modern day medicine (Piddock, 2012), and the use of antibiotics impacts on various medical procedures such as cancer treatment, organ transplant, knee and hip replacement surgeries. However, successful antibacterial therapy is threatened by resistant bacterial strains. With the prevalence of resistant bacteria increasing and the decline in the rate of discovery of new antibiotics, alternative approaches such as host directed anti-infective therapy, the use of antibacterial agents that exhibit synergism with the immune response or the use of adjuvant or combination antibiotic therapy should be exploited (Anuforom et al., 2014). However, one caveat to the use of antibacterial immunomodulatory agents is the need to discover a balance that not only generates an immune response sufficient to supress infection, but also capable of limiting any inflammatory response that can damage the host. Antibiotics that can modulate expression of TLR may also drive an exacerbated inflammatory response (Bode et al., 2014, O'Neill et al., 2009). It will be necessary for detailed mechanistic studies to be carried out to provide a better understanding of the dynamics of any such therapy (Hussell, 2012) .

Table 7.1 Summary of findings for effects of antibiotics on innate immune cells functions +/- infection by pathogenic wild type *Salmonella* SL1344

	Effect of antibiotics on;							
	***Salmonella* in liquid medium**	***Salmonella* Adhesion**	***Salmonella* Invasion**	**Phagocytosis**	**Oxidative burst**	**Bacterial killing**	**TLR gene expression**	**Cytokine gene expression**
Ciprofloxacin	Reduced viability of *Salmonella* at C_{max} concentration	Increased adhesion	Decreased at C_{max}	Decreased	No effect	No effect	Decreased expression of TLR 3,4,5,8	Increased expression of IL1b, IL6 and TNFα
Ceftriaxone	Inhibited growth of *Salmonella* at C_{max} concentration	Increased adhesion	Decreased at MIC	No effect	Increased	Concentration dependent increased killing	Decreased expression of TLR 3,4,5,8. Increased expression of TLR 2	Increased expression of IL1b, IL6 and TNFα
Tetracycline	No effect	No effect	No effect at both concentrations	No effect	No effect	No effect		
Azithromycin	No effect	Increased adhesion at C_{max}, decreased adhesion at MIC	Increased at MIC, decreased at C_{max}	No effect	No effect	No effect		
Streptomycin	No effect	Increased adhesion at	No effect at both			No effect		

		C_{max}	concentrations			

Overall, my findings have shown that antibiotics are able to influence four processes during bacterial interaction with the host; neutrophil phagocytosis and oxidative burst, bacterial adhesion and invasion, as well as expression of genes in the TLR signalling pathway. The findings of my thesis illustrate that beyond their conventional use, antibiotics can alter the host's innate immune responses to, and interaction with bacteria. This additional property of antibiotics can be explored and optimised for increased efficacy of antimicrobial therapy.

7.3   Key findings of research

- In response to bacteria, neutrophil oxidative burst was increased when neutrophils were exposed to ceftriaxone, with a corresponding increase in bacterial killing of SL1344. This confirms previous findings by Wenisch et al., (1995), where they reported improved phagocytic function in neutrophils following ceftriaxone treatment.

- Phagocytosis was decreased in neutrophils exposed to ciprofloxacin, although this had no effect on the killing of *Salmonella* by these cells. This is contradictory to a previous study that shows that ciprofloxacin did not induce any significant effect on neutrophil functions (Boogearts et al., 1986).

- My data is the first study to show an increase in adhesion and invasion levels for *Salmonella* SL1344 to antibiotic treated J774 and murine bone marrow derived macrophages, compared to untreated macrophages. However, antibiotic treatment did not induce an increase in invasion of *Salmonella* SL1344 to J774 macrophages.

- This is the first study to show dissimilarities between adhesion numbers of *S.* Typhimurium to human macrophages compared to murine macrophages. My findings show that while bacterial adhesion to human macrophages was not altered in antibiotic pre-treated or untreated macrophages, murine macrophages responded with increased adhesion following treatment. This is in line with current debate on the use of mouse models on the study of human disease, and the challenge in translating findings of *in vitro* experiments to animal models and to human diseases (Seok et al., 2013).

- My data is the first to show that expression of genes encoding TLR signalling and cytokine production of J774 macrophages was not altered when macrophages were exposed to ciprofloxacin or ceftriaxone alone. TLR gene expression was generally decreased or unaffected with only TLR2 expression showing an increase, a response that was not further affected by antibiotic treatment. Bode et al shown similar results with moxifloxacin down regulating TLR4 and TLR6, but no effect on TLR2 expression in THP-1 cells stimulated with LPS for 24 hours. Therefore although the methodology was significantly different similar results were shown.

- The current study is one of the first to investigate antibiotic pre-treatment of immune cells to determine the effect of *S.* Typhimurium challenge. Several studies have reported *in vivo* effects of antibiotic pre-treatment, resulting in intestinal infection. However, this was due to changes to the gut microbiome and effects on immune cells were not described (Barthel et al., 2003, Hapfelmeier and Hardt, 2005)

7.4   Suggested further work

The results presented in this thesis demonstrate that antibiotics can alter the interactions between innate immune cells and bacteria. Further studies to investigate the underlying mechanisms by which antibiotics affect this interaction should be exploited. These include identifying the subcellular localisation and distribution of antibiotics in the various compartments of neutrophils and macrophages, following exposure to these agents and investigate how subcellular functions of immune cells are influenced by the presence of these antibiotics using fluorescently labelled antibacterial agents. As *S.* Typhimurium causes less severe disease in humans,

further work investigating adhesion and invasion of the human adapted *S.* Typhi to human immune cells should be carried out to understand whether the discrepancies between bacterial adhesion to THP-1 cells and monocyte derived macrophages used in this study are *S.* Typhimurium specific.

This study investigated the effects of antibiotics on the TLR signalling pathways of J774 macrophages in response to *S.* Typhimurium infection and antibiotic treatment showed that some antibiotics altered expression of genes responsible for TLR signalling and cytokine production. To better understand whether these results are comparable for human infections, studies should be carried out to explore the gene expression profile using human adapted *S.* Typhi on human macrophage cells. Furthermore, while gene expression data in this study provides some insight into the effect of antibiotics on specific signalling pathway, I would like to have carried out metabolomic profiling of macrophages in response to *Salmonella* infection and antibacterial exposure for a holistic understanding of the complete changes in the physiology of immune cells.

I showed that IL-1β mRNA expression was increased in *Salmonella* infected macrophages pre-treated with ciprofloxacin. As IL-1β is produced downstream of the inflammasome activation through TLR signalling in response to bacterial LPS, binding of TLR to TLR ligands such as LPS activates the transcription of pro-IL-1β. Activation of Caspase-1 cleaves pro IL-1β to mature IL-1β, which is secreted out of the cell. I would like to have investigated the effect of ciprofloxacin on activation of the inflammasome by measuring the effect of this drug on the activation of Caspase-1. My data also showed that IL-1β protein production was not consistent with its mRNA expression. This may be due to reasons such as pro IL-1β not cleaved to

mature IL-1β or that although this cytokine may be produced, it is not secreted out of the cell. Hence, it would be interesting to investigate and quantify intracellular production of cytokines within macrophages in response to antibiotic pre-treatment and SL1344 infection using a protein transport inhibitor (e.g. Brefeldin A), which prevents proteins from being exported out of the cell.

Appendix 1. Conference presentations associated with this thesis

Anuforom, O., Wallace, G.R. and Piddock, L.J.V. Antibiotics of different classes influence the immune response to bacteria. 24th ECCMID, Barcelona, 2014, abstract reference no: ECCMI-0631.

Anuforom, O., Wallace, G.R. and Piddock, L.J.V. Antibiotics of different classes influence the immune response to bacteria. University of Birmingham Post Graduate Research Conference, June 2014.

Anuforom, O., Wallace, G.R. and Piddock, L.J.V. Do antibiotics affect the immune response? Institute of Microbiology and Infection (IMI) Forum, University of Birmingham, September 2013

Publications

Anuforom, O., Wallace, G.R. and Piddock, L.J.V. 2014 The immune response and antibacterial therapy. *Medical Microbiology and Immunology*, DOI 10.1007/s00430-014-0355-0.

Original article in preparation

Anuforom, O., Wallace, G.R. and Piddock, L.J.V. Effect of antibiotic exposure on the TLR signalling pathway of J774 macrophages in response to *Salmonella* Typhimurium infection.

Appendix 2. Fold change data

Fold change in expression of genes in the TLR signalling pathway for ciprofloxacin treated and untreated macrophages infected with *Salmonella*.

		Fold Change (comparing to control group)					
		Macrophage + Cip		Macrophage + Cip +SL1344		Macrophage + SL1344	
		Fold Change	Comments	Fold Change	Comments	Fold Change	Comments
A01	Agfg1	0.781	OKAY	0.7303	OKAY	0.8736	OKAY
A02	Btk	0.839	OKAY	0.3264	OKAY	0.3043	A
A03	Casp8	0.9096	OKAY	0.4611	OKAY	0.3812	OKAY
A04	Ccl2	0.8971	OKAY	2.7897	OKAY	2.4384	OKAY
A05	Cd14	1.1173	OKAY	1.1362	OKAY	1.0075	OKAY
A06	Cd80	1.021	B	16.1219	OKAY	8.5199	OKAY
A07	Cd86	1.0353	OKAY	1.5962	OKAY	1.6312	OKAY
A08	Cebpb	0.8487	OKAY	1.5136	OKAY	1.9321	OKAY
A09	Chuk	0.9352	OKAY	0.7617	OKAY	0.7821	OKAY
A10	Clec4e	0.9417	OKAY	2.0965	OKAY	2.2179	OKAY
A11	Csf2	0.727	B	4.4147	A	3.6035	B
A12	Csf3	0.933	C	26.5795	A	47.9172	A
B01	Cxcl10	0.6659	OKAY	6.36	OKAY	4.9049	OKAY
B02	Eif2ak2	0.839	OKAY	0.6691	OKAY	0.4379	OKAY
B03	Elk1	0.8198	B	0.3707	OKAY	0.3754	OKAY
B04	Fadd	0.772	OKAY	0.241	A	0.2296	A
B05	Fos	0.8971	OKAY	0.336	OKAY	0.4099	A
B06	Hmgb1	0.9571	OKAY	0.4798	OKAY	0.47	OKAY

B07	Hras1	0.8467	OKAY	0.3494	OKAY	0.2637	OKAY
B08	Hspa1a	0.933	C	0.7089	C	1.0678	C
B09	Hspd1	0.7492	OKAY	0.5452	OKAY	0.5419	OKAY
B10	Ifnb1	0.9159	B	11.3296	A	10.5055	A
B11	Ifng	0.933	C	0.7089	C	1.0678	C
B12	Ikbkb	0.8048	OKAY	0.4788	OKAY	0.445	OKAY
C01	Il10	0.8746	B	3.2615	A	1.268	B
C02	Il12a	0.933	C	0.7089	C	1.0678	C
C03	Il1a	0.9772	B	96.6715	A	32.5392	A
C04	Il1b	0.9659	B	118.6044	A	38.96	A
C05	Il1r1	0.6958	B	0.5286	OKAY	0.7963	B
C06	Il2	0.933	C	0.7142	B	1.0678	C
C07	Il6	0.933	C	143.4968	A	43.0059	A
C08	Il6ra	0.8123	OKAY	0.1822	A	0.1645	A
C09	Irak1	0.8236	OKAY	0.4564	OKAY	0.4578	OKAY
C10	Irak2	0.8685	OKAY	1.6887	OKAY	2.0759	OKAY
C11	Irf1	0.7614	OKAY	0.8778	OKAY	0.3543	OKAY
C12	Irf3	0.9482	OKAY	0.4655	OKAY	0.4664	OKAY
D01	Jun	0.9287	OKAY	0.5316	OKAY	0.8817	OKAY
D02	Lta	0.933	C	1.4404	B	2.0122	B
D03	Ly86	1.0968	OKAY	0.4849	OKAY	0.4658	OKAY
D04	Ly96	0.8566	OKAY	0.6308	OKAY	0.6003	OKAY
D05	Map2k3	0.8293	OKAY	0.4155	OKAY	0.3717	OKAY
D06	Map2k4	0.8428	OKAY	0.6282	OKAY	0.6414	OKAY
D07	Map3k1	0.8255	OKAY	0.5255	OKAY	0.717	OKAY
D08	Map3k7	0.8085	OKAY	0.4796	OKAY	0.5698	OKAY
D09	Mapk8	0.8104	OKAY	0.5731	OKAY	0.6739	OKAY

D10	Mapk8ip3	0.6926	B	0.7225	OKAY	0.6831	OKAY
D11	Mapk9	0.8685	OKAY	0.4658	OKAY	0.4907	OKAY
D12	Muc13	0.933	C	0.7089	C	1.0678	C
E01	Myd88	0.683	B	0.512	B	0.4656	OKAY
E02	Nfkb1	0.837	OKAY	1.5247	OKAY	1.7483	OKAY
E03	Nfkb2	0.7354	B	1.3306	OKAY	1.2918	OKAY
E04	Nfkbia	0.9885	OKAY	4.12	OKAY	4.8747	OKAY
E05	Nfkbib	0.5932	B	1.1638	B	1.5302	B
E06	Nfkbil1	0.722	OKAY	1.0694	OKAY	0.9446	OKAY
E07	Nfrkb	0.8827	OKAY	0.5173	OKAY	0.5265	OKAY
E08	Nr2c2	0.9138	OKAY	0.3724	OKAY	0.4293	OKAY
E09	Peli1	0.8566	OKAY	2.7146	OKAY	2.7796	OKAY
E10	Pglyrp1	0.7304	B	0.4171	OKAY	0.5016	B
E11	Ppara	0.933	C	0.7089	C	1.0678	C
E12	Ptgs2	0.8566	OKAY	15.1029	OKAY	23.2809	OKAY
F01	Rel	0.8746	OKAY	2.2657	OKAY	3.231	OKAY
F02	Rela	0.8141	OKAY	0.5105	OKAY	0.6538	OKAY
F03	Ripk2	1.0023	OKAY	2.6774	OKAY	3.5605	OKAY
F04	Tbk1	0.8217	OKAY	0.4387	OKAY	0.5904	OKAY
F05	Ticam1	0.7492	B	1.0158	A	1.9092	A
F06	Ticam2	0.9682	B	1.6423	OKAY	2.1599	OKAY
F07	Tirap	0.816	OKAY	0.4144	A	0.3256	A
F08	Tlr1	0.9794	A	1.4701	A	1.563	A
F09	Tlr2	0.8409	OKAY	1.6328	OKAY	1.6411	OKAY
F10	Tlr3	0.7631	OKAY	0.5526	OKAY	0.3643	A
F11	Tlr4	0.8448	OKAY	0.1837	OKAY	0.2006	OKAY
F12	Tlr5	0.9908	B	0.1009	OKAY	0.1014	OKAY

G01	Tlr6	0.8467	B	0.6154	OKAY	0.6297	B
G02	Tlr7	0.9548	OKAY	0.4802	OKAY	0.5681	OKAY
G03	Tlr8	0.8706	OKAY	0.1165	A	0.0503	A
G04	Tlr9	0.8746	OKAY	0.6114	OKAY	0.685	OKAY
G05	Tnfα	0.9013	OKAY	6.999	OKAY	21.5995	OKAY
G06	Tnfaip3	0.8566	A	4.952	OKAY	6.09	OKAY
G07	Tnfrsf1a	0.9117	OKAY	0.4242	OKAY	0.5201	OKAY
G08	Tollip	0.839	OKAY	0.5627	OKAY	0.7504	OKAY
G09	Tradd	1.0546	OKAY	0.5064	OKAY	0.4375	OKAY
G10	Traf6	0.9704	OKAY	0.8382	OKAY	0.9201	OKAY
G11	Ube2n	0.7236	OKAY	0.4915	OKAY	0.4115	OKAY
G12	Ube2v1	0.8665	B	0.4695	OKAY	0.7493	B
H01	Actb	0.8726	OKAY	0.6401	OKAY	0.8936	OKAY
H02	B2m	1	OKAY	1	OKAY	1	OKAY
H03	Gapdh	0.9244	OKAY	0.8551	OKAY	1.0958	OKAY
H04	Gusb	0.837	OKAY	0.5249	OKAY	0.5837	OKAY
H05	Hsp90ab1	0.8274	OKAY	0.5692	OKAY	0.6793	OKAY
H06	MGDC	0.933	C	0.7089	C	1.0678	C
H07	RTC	1.0595	B	1.2052	B	1.6746	B
H08	RTC	1.1975	B	1.3572	B	2.0878	B
H09	RTC	1.0187	B	0.9211	B	1.4695	B
H10	PPC	0.8606	OKAY	0.6391	OKAY	0.9523	OKAY
H11	PPC	0.8992	OKAY	0.6736	OKAY	1.0297	OKAY
H12	PPC	0.9013	OKAY	0.6931	OKAY	1.0218	OKAY

Fold change in expression of genes in the TLR signalling pathway for ceftriaxone treated and untreated macrophages infected with *Salmonella*.

		Fold Change (comparing to control group)					
		Macrophage + Cef		Macrophage + SL1344 + Cef		Macrophage + SL1344	
		Fold Change	Comments	Fold Change	Comments	Fold Change	Comments
A01	Agfg1	0.8397	B	0.8011	A	1.1244	A
A02	Btk	1.1794	B	0.7002	OKAY	0.6996	OKAY
A03	Casp8	0.9535	A	0.581	OKAY	0.6088	A
A04	Ccl2	1.0291	OKAY	3.7472	OKAY	1.7275	OKAY
A05	Cd14	0.7045	OKAY	1.7738	OKAY	1.4294	OKAY
A06	Cd80	1.041	C	3.017	OKAY	2.3023	OKAY
A07	Cd86	1.1551	OKAY	1.9834	OKAY	1.4755	OKAY
A08	Cebpb	1.2553	OKAY	3.3291	OKAY	3.6045	OKAY
A09	Chuk	0.9557	OKAY	0.9549	OKAY	0.8595	OKAY
A10	Clec4e	0.8187	B	4.511	A	4.9695	A
A11	Csf2	0.9624	B	2.6877	OKAY	1.1266	B
A12	Csf3	1.041	C	0.5278	B	0.6672	C
B01	Cxcl10	1.549	B	44.4955	A	25.2362	A
B02	Eif2ak2	0.9317	B	0.475	OKAY	0.4864	OKAY
B03	Elk1	1.0802	B	0.7774	B	0.6503	B
B04	Fadd	1.3177	B	0.6605	OKAY	0.6085	OKAY
B05	Fos	1.2495	B	0.5513	B	0.7272	B
B06	Hmgb1	1.0172	OKAY	0.7946	OKAY	0.7565	OKAY
B07	Hras1	1.1183	OKAY	0.6864	OKAY	0.6326	OKAY

B08	Hspa1a	1.041	C	0.4293	C	0.6672	C
B09	Hspd1	0.9803	OKAY	0.8375	OKAY	1.0834	OKAY
B10	Ifnb1	0.9513	B	1.2016	B	0.6789	B
B11	Ifng	1.041	C	0.4293	C	0.6672	C
B12	Ikbkb	1.4722	B	1.2602	OKAY	1.277	B
C01	Il10	1.041	C	0.4293	C	0.6672	C
C02	Il12a	1.041	C	0.4293	C	0.6672	C
C03	Il1a	1.041	C	21.051	A	2.2159	B
C04	Il1b	1.041	C	127.1405	A	15.505	OKAY
C05	Il1r1	1.041	C	0.4293	C	0.6672	C
C06	Il2	1.041	C	0.4293	C	0.6672	C
C07	Il6	1.041	C	3.6846	OKAY	0.8461	B
C08	Il6ra	1.1794	B	0.4499	OKAY	0.546	B
C09	Irak1	1.4621	OKAY	0.99	B	1.0856	B
C10	Irak2	0.7481	B	4.321	A	4.4982	A
C11	Irf1	1.3864	B	1.5508	OKAY	1.3515	B
C12	Irf3	1.1524	OKAY	0.7335	OKAY	0.6913	OKAY
D01	Jun	0.7516	OKAY	0.8305	OKAY	1.2813	OKAY
D02	Lta	1.041	C	0.4293	C	0.6672	C
D03	Ly86	0.9736	OKAY	0.6894	OKAY	0.5278	OKAY
D04	Ly96	1.1631	B	0.7827	A	0.7809	B
D05	Map2k3	1.0777	B	0.9611	A	0.8001	B
D06	Map2k4	1.0338	B	0.9909	B	0.9566	B
D07	Map3k1	1.2437	B	0.8655	B	1.339	OKAY
D08	Map3k7	1.2238	B	0.9582	A	0.9767	B
D09	Mapk8	0.7908	OKAY	0.8413	OKAY	0.8793	OKAY
D10	Mapk8ip3	1.1209	B	0.7764	B	0.8924	B

D11	Mapk9	0.7889	B	0.6817	A	0.6826	OKAY
D12	Muc13	1.041	C	0.4293	C	0.6672	C
E01	Myd88	1.611	B	1.4829	B	1.3497	B
E02	Nfkb1	1.0629	OKAY	2.5791	OKAY	2.2653	OKAY
E03	Nfkb2	1.041	B	1.4823	B	1.4184	B
E04	Nfkbia	0.5401	A	7.1466	OKAY	6.0066	OKAY
E05	Nfkbib	0.8339	B	0.5951	B	0.7158	B
E06	Nfkbil1	0.9826	B	1.9298	OKAY	1.2753	B
E07	Nfrkb	1.0978	B	0.834	B	1.0407	B
E08	Nr2c2	1.0928	B	0.7869	B	0.8555	B
E09	Peli1	0.9382	B	3.3431	A	2.4109	A
E10	Pglyrp1	0.813	B	0.3644	OKAY	0.521	B
E11	Ppara	1.041	C	0.4293	C	0.6672	C
E12	Ptgs2	1.2437	B	32.5202	A	30.0742	A
F01	Rel	1.1313	B	7.4563	A	7.7482	A
F02	Rela	1.0953	B	1.9331	OKAY	1.7899	OKAY
F03	Ripk2	0.5592	OKAY	2.8201	A	3.028	A
F04	Tbk1	0.9469	B	1.116	B	1.0627	B
F05	Ticam1	1.5743	B	2.8455	OKAY	1.9318	OKAY
F06	Ticam2	0.9513	B	1.4097	B	1.4287	B
F07	Tirap	1.4453	OKAY	0.9835	B	0.9379	B
F08	Tlr1	0.6204	B	1.2705	B	1.311	B
F09	Tlr2	0.9126	B	5.5229	A	6.5604	A
F10	Tlr3	0.9	B	0.4138	OKAY	0.44	OKAY
F11	Tlr4	1.0032	B	0.4894	OKAY	0.4271	OKAY
F12	Tlr5	1.432	OKAY	0.1835	OKAY	0.2359	OKAY
G01	Tlr6	2.1307	OKAY	1.7905	B	1.7881	B

G02	Tlr7	0.7061	A	0.679	OKAY	0.8317	OKAY
G03	Tlr8	0.8693	A	0.2072	A	0.2061	A
G04	Tlr9	0.7293	B	1.4636	A	1.5225	B
G05	Tnfα	0.8534	B	32.7072	A	27.4123	A
G06	Tnfaip3	1.0728	B	15.7419	A	15.7667	OKAY
G07	Tnfrsf1a	0.9	B	0.7325	B	0.6086	B
G08	Tollip	1.1524	A	1.0114	A	0.9919	A
G09	Tradd	1.1157	OKAY	0.6445	OKAY	0.6533	OKAY
G10	Traf6	0.8436	B	1.1097	B	1.1143	B
G11	Ube2n	1.0777	B	0.6246	B	0.6713	B
G12	Ube2v1	1.041	C	0.5237	B	0.7469	B
H01	Actb	0.9361	OKAY	1.0365	OKAY	0.9715	OKAY
H02	B2m	0.8187	OKAY	1.0236	OKAY	0.9793	OKAY
H03	Gapdh	1.0196	OKAY	1.0858	OKAY	1.0459	OKAY
H04	Gusb	1.2153	OKAY	0.89	OKAY	0.9022	OKAY
H05	Hsp90ab1	1.0531	OKAY	0.9753	OKAY	1.1139	OKAY
H06	MGDC	1.041	C	0.4293	C	0.6672	C
H07	RTC	0.4913	B	0.1356	B	0.8669	B
H08	RTC	0.5039	B	0.2327	B	0.912	B
H09	RTC	0.5489	OKAY	0.2805	OKAY	1.4991	B
H10	PPC	1.1578	OKAY	0.453	OKAY	0.634	OKAY
H11	PPC	1.0243	OKAY	0.0046	OKAY	0.6052	OKAY
H12	PPC	0.9339	OKAY	0.3985	OKAY	0.5325	OKAY

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