Cationic Host Defence Peptides as novel therapeutics for *Chlamydia* infection

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Abstract

Chlamydia represents a group of Gram-negative bacteria which can infect and cause disease in a diverse range of organisms. The most well-known member of the family is *Chlamydia trachomatis*, the most common bacterial sexually transmitted infection across the world, which is associated with reproductive complications including infertility and miscarriage. In addition, other emerging *Chlamydia*-like organisms such as *Waddlia chondrophila are proposed to* represent a health threat, as their association with several human diseases is being investigated. As human vaccines for these infections are not currently available and antibiotic resistance may potentially risk the current treatments over the coming years, the discovery of new effective antimicrobial drugs is essential.

Cationic Host Defence Peptides (CHDPs) are known for their key role in the innate immune response. In mammals, there are two main families of CHDPs, cathelicidins and defensins. These peptides have been identified in several different cell types including epithelial cells, macrophages and neutrophils among others. The human cathelicidin, LL-37, displays broad spectrum antimicrobial and immunomodulatory potential following proteolytic cleavage from the precursor molecule hCAP-18. In addition to their direct antimicrobial activity, cathelicidins have been shown to influence the inflammatory response to pathogens and to play a role in wound repair and immune cell recruitment and differentiation. As such, they represent exciting prospects for the development of novel peptide-based therapeutics for a range of infections.

This study investigated the activity of cathelicidins from humans and animals against different *Chlamydia* and *Waddlia chondrophila*, assessing changes in bacterial population as well as immunomodulatory effects on the epithelial host cell line, HEp2, during infection. Our results show that cathelicidins can have a direct effect on *Chlamydia* populations and that the effect is most visible when the pathogen is directly exposed to the peptide. We demonstrate that cathelicidins can directly modulate the host cell response to these infections, likely through direct inhibition of the pathogen induced inflammatory response, rather than by modulation of host cell death pathways. Furthermore, we demonstrate that the emerging pathogen *Waddlia chondrophila* appears to show intrinsic resistance to the activity of these peptides, potentially representing a concerning trait of this organism in terms of innate immune evasion.

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Commonly used abbreviations

CHDP – Cationic Host Defence Peptide

HDP – Host Defence Peptide

hCAP18 – Human cationic antimicrobial protein 18

PRR – Pattern recognition receptor

TLR – Toll-like receptor

MIP – Macrophage infectivity potentiator

HSP60 – Heat shock protein 60

LPS – Lipopolysaccharide

MOMP – Major outer membrane porin protein

T3SS – Type III secretion system

EB – Elementary body

RB – Reticulate body

LGV – Lymphogranuloma venereum

L2 – LGV II serovar

PAMP – Pathogen associated molecular pattern

MyD88 – Myeloid differentiation primary response protein 88

TNF-α – Tumour necrosis factor alpha

TRAF-6 – TNF receptor associated factor 6

NLR – NOD-like receptors

NOD – Nucleotide-binding olimerization domain

IL – Interleukin

PG – Protegrin

SMAP – Sheep myeloid antimicrobial peptide

BMAP – Bovine myeloid antimicrobial peptide

PBS – Phosphate buffer saline

IMDM – Iscove's modified Dulbecco's media

FBS – Foetal bovine serum

sLL-37 – Scrambled LL-37

HEp2 – Human epithelial cells 2

MOI – Multiplicity of infection

BSA – Bovine serum albumin

TRITC – Tetramethylrhodamine

GFP – Green fluorescent protein

DAPI – 4′,6-diamidino-2-phenylindole

LDH – Lactose dehydrogenase

μM – Micromolar

μl – Microlitre

ml – Millilitre

μg – Microgram

1. Introduction

1.1 Chlamydiae

Chlamydiae are Gram-negative, obligate intracellular pathogens of diverse host organisms, including humans (Bachmann, Polkinghorne and Timms, 2014). Chlamydiae are the agents of the most common bacterial sexually transmitted disease as well as other infections, such as pneumonia, psittacosis, trachoma, and lymphogranuloma venereum. They lack the ability to produce sufficient energy to grow independently, and therefore can only complete their life cycle inside a host cell. The best-studied group of the Chlamydiae phylum is the Chlamydiaceae family, which comprises 11 species that are pathogenic to humans or animals, such as *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Stephens *et al.*, 1999).

1.1.1 Life cycle

1.1.1.1 Chlamydiae recognition and internalization

Infection starts when the infectious elementary body attaches to the epithelial host cell membrane by the formation of a trimolecular bridge between the bacterial adhesins, the host heparan sulphate (like glycosaminoglycan, GAG) and host receptors (Mehlitz and Rudel, 2013). This binding is thought to be a two-step process for some species, involving an initial reversible interaction with host heparan sulphate-like glycosaminoglycans, followed by the high affinity irreversible binding to host cell receptors (Dautry-Varsat, Subtil and Hackstadt, 2005). Bacterial adhesins are recognised by pattern recognition receptors (PRR), including Toll-like receptors (TLRs). Some chlamydial components have been found to be implicated in TLR-dependent cell activation: the lipoprotein macrophage infectivity potentiator (MIP), lipopolysaccharide (LPS) and the chlamydial heat shock protein (HSP60). Other ligands that have been also found to activate TLR signalling pathways in *Chlamydia*, are chlamydial major outer membrane porin proteins (MOMP). Other examples of receptors implicated in the recognition of chlamydial PAMPs are mannose-receptors, Epidermal Growth Factor Receptor (EGFR) and estrogen receptor complex. When the bacteria have attached to the host cell membrane, pre-

synthesized type III secretion system (T3SS) effectors are injected in the host cell, which allows for cytoskeletal rearrangement to facilitate the bacterial internalisation and the establishment of an anti-apoptotic state in the host cell. Of note, Chlamydiae are able to induce small GTPase-dependent reorganization of the actin cytoskeleton to promote bacterial entry (Dautry-Varsat, Subtil and Hackstadt, 2005; Kumar and Valdivia, 2008). The pathogen is then internalised in a membrane-bound vesicle, known as the inclusion.

1.1.1.2 Establishment of the inclusion and multiplication

Bacteria in the phylum Chlamydiae are characterised by their complex intracellular developmental cycles. All species belonging to this phylum share a biphasic life cycle in which they alternate between an infectious elementary body (EB) and the intracellular non-infectious reticulate body (RB) (Figure 1). Once the elementary body has entered the host cell in the vesicle-like compartment, early chlamydial gene expression will result in avoidance of the natural endolysosomal pathway and in transportation through the microtubules network to the microtubule organizing center (MTOC) (Scidmore, 2011). Inside the inclusion, the elementary body transits to the reticulate body and bacterial protein synthesis is initiated. To ensure essential host lipid and protein acquisition by the bacterial inclusion, newly secreted inclusion membrane proteins promote the redirection of host membrane trafficking vesicles into the inclusion. The reticulate body then replicates exponentially and continues producing more effectors to modulate host cell processes. Under *in vitro* stress conditions, chlamydial inclusions from species of the Chlamydiaceae family can enter in a persistent state called aberrant body. In this state, replication and mRNA synthesis continue, as well as an aberrant enlargement of the RBs. Therefore, cell division is altered, and is not resumed until the stress has been removed (Wyrick, 2010). While the molecular mechanism by which *Chlamydia* enters and exits persistence is not known, growing evidence supports the observation that this stage also occurs *in vivo* (Hogan *et al.*, 2004; Schoborg, 2011). The late stages of the infection also involve the transition from reticulate bodies to elementary bodies, after 48-72 hours post-infection, and the elementary bodies then exit the host cell.

1.1.1.3 Mechanisms of host cell exit

Chlamydiae exit occurs by two exclusive pathways: lysis of the inclusion and host cell membrane and a packaged release process, called extrusion (Hybiske and Stephens, 2007). Both pathways take place at similar frequencies. In the case of the first mechanism, the spontaneous rupture of the inclusion membrane is followed by the progressive permeabilisation of intracellular compartments, ending with the permeabilisation of the host cell plasma membrane. These permeabilisations are mostly caused by the activity of cell and bacterial proteases (Hybiske and Stephens, 2007). The second exit pathway, extrusion, consists in the release of the new elementary bodies inside a membrane compartment, leaving the host cell intact. This mechanism of dissemination allows the *Chlamydia* to go undetected from local immune responses, preventing the release of inflammatory contents from the host cell.

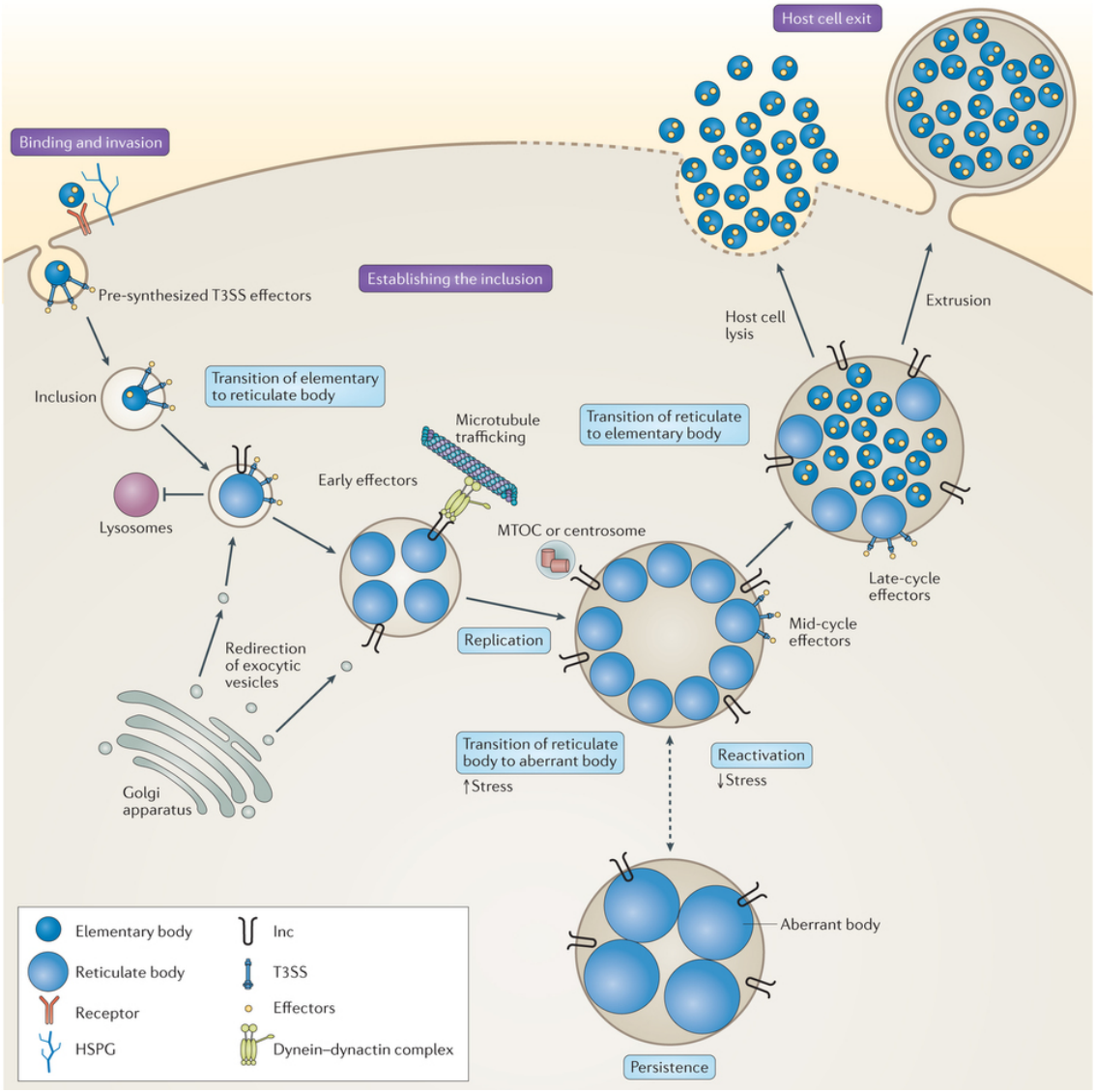


Figure 1. The life cycle of *Chlamydia trachomatis*. (Elwell, Mirrashidi and Engel, 2016). Elementary bodies bind to the host cell by the formation of a trimolecular bridge between bacterial adhesins, host receptors and host heparan sulfate proteoglycans (HSPGs). Pre-synthesized type III secretion system (T3SS) effectors are then injected into the host cell to facilitate chlamydial entry and to promote the establishment of an anti-apoptotic state. The elementary body is internalised into a membrane-bound compartment, the inclusion, which rapidly dissociates from the canonical endocytic pathway. Next, elementary bodies convert to reticulate bodies and newly secreted inclusion membrane proteins (Incs) promote nutrient acquisition by redirecting host cell exocytic vesicles from the Golgi apparatus. During the late stages of infection, reticulate bodies differentiate back to elementary bodies. Finally, elementary bodies will exit the host through extrusion or lysis.

1.1.2 Chlamydia trachomatis

Chlamydia trachomatis is the causative agent of several acute and chronic, as well as local and systemic, human diseases such as trachoma, urogenital and neonatal infections. *C. trachomatis* was first identified in 1907 by Halberstaedter and von Prowazec from conjunctival scrapings from subjects with trachoma. *C. trachomatis* was initially thought to be protozoa and later a virus, before being correctly identified as intracellular bacteria. Because chlamydial infection was first identified in the eye and due to its broad range of symptoms, or lack of them, the infection was not thought to be sexually transmitted until 1976 (Schachter, Causse and Tarizzo, 1976). *C. trachomatis* strains are divided into three biovars, variants that possess physiological and/or biochemical differences from other strains, and further subdivided into serovars, which are subdivisions of subspecies characterized by a differential set of antigens. The trachoma biovar comprises the serovars A-C and is the leading cause of non-congenital blindness in Western countries, whereas the genital tract biovar (serovars D-K) is the most common bacterial sexually transmitted infection worldwide. Serovars L1-L3 belong to the lymphogranuloma venereum (LGV) biovar and cause invasive urogenital and anorectal infection.

1.1.2.1 Clinical manifestations

The various *C. trachomatis* serovars are responsible of a variety of clinical conditions in humans. The trachoma biovar is the leading cause of infectious blindness worldwide

(Resnikoff *et al.*, 2008). Although the genital serovars of *C. trachomatis* can infect the ocular tissues, it is unlikely that it will lead to blinding sequelae. Regarding the ocular manifestations caused by the trachoma biovar, patients with repeated conjunctival infections suffer from chronic inflammation of the conjunctiva and follicle formation on its surface. The increasing roughness of the conjunctiva and its scarring, leads to cornea scarring which can result in blindness if the infection continues untreated. The clinical manifestations of trachoma change with age, being more active at young ages.

Lymphogranuloma venereum (LGV) is a sexually transmitted disease that primarily attacks lymphatic and subepithelial tissues of the genital tract, causing primary lesions. The early stage of the infection involves painless genital papulae and pustules and is normally asymptomatic. Infection of the lymphatic tissue induces a lymphoproliferative reaction (Ceovic and Gulin, 2015). LGV infection travels by lymphatics to the lymph nodes, where it can infect macrophages and results in systemic disease in the last stage of the infection. LGV disease is caused predominantly by the serovar L2.

Urogenital infections caused by *C. trachomatis* normally affect the urethra and cervix in women and urethra in men. *C. trachomatis* urogenital infection can go undiagnosed as it displays higher percentages of asymptomatic infections: 50 per cent of the infected men are asymptomatic, whereas in women this percentage is even higher, approximately 70-80 per cent (Malhotra *et al.*, 2013). Asymptomatic infections can remain undetected, which can lead to major complications and a delayed treatment. Untreated infections in females can cause pelvic inflammatory disease (Paavonen and Eggert-Kruse, 1999) that can lead to infertility (Haggerty *et al.*, 2010) and ectopic pregnancy (Menon *et al.*, 2015). *C. trachomatis* role in miscarriage has been proposed, though the possible link and mechanism is still poorly understood (Giakoumelou *et al.*, 2016).

1.1.2.2 Host innate immune response

Chlamydia is recognized in epithelial cells by cell surface receptors, endosomal receptors and cytosolic innate immune receptors (Elwell, Mirrashidi and Engel, 2016). These receptors recognize conserved pathogen-associated molecular patterns found in certain microorganisms and are expressed not only in epithelial but also in dendritic cells and

innate immune cells, such as macrophages and neutrophils. Chlamydial pathogen-associated molecular patterns (PAMPs) recognition is highly complex. On binding, the pathogen activates the PRRs (predominantly Toll-like receptors). TLRs recognize microbial infection and have an essential role in the induction of innate and adaptive immune responses. TLR4 recognizes bacterial lipopolysaccharide (LPS), whereas TLR2 detects bacterial lipoproteins and glycolipids. The implications of more than one Toll-like receptor were studied using knock-out mice for TLR4 and TLR2 in *Chlamydia muridarum* infection (Entrican *et al.*, 2004; Imtiaz *et al.*, 2006). Chlamydial infection is recognized predominantly by TLR2 (Wang *et al.*, 2017). Among the reported molecules responsible of innate immune response activation via TLR in *Chlamydia* are MIP (macrophage infectivity potentiator), which is recognized via TLR2/TLR1/TLR6, and CD14 and cHSP60 (Heat Shock Protein 60), recognized both via TLR4 and TLR2. Other ligands activating TLR2 signaling pathway in *Chlamydia* infection are chlamydial major outer membrane porin proteins (MOMP), TLR1 playing a co-receptor role in its recognition (Massari *et al.*, 2013). TLR activation leads to the induction of the innate immune response and to pro-inflammatory cytokine production due to a signaling cascade that involves the myeloid differentiation primary response protein 88 (MyD88) and tumour necrosis factor alpha (TNF-α) receptor associated factor 6 (TRAF-6) (Figure 2).

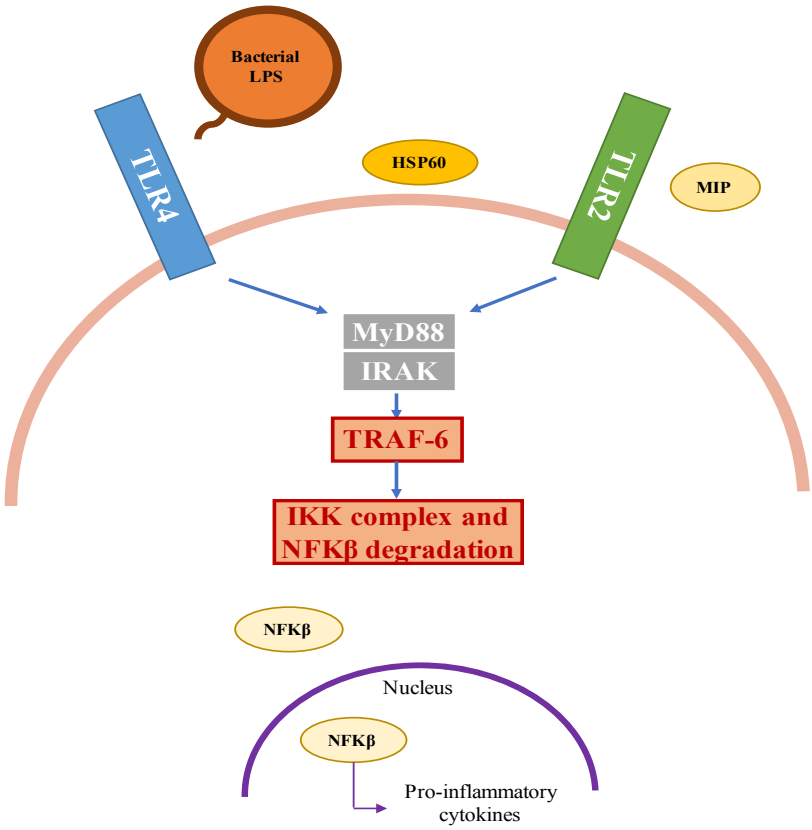


Figure 2. TLR2 and TLR4 dependent inflammatory responses during chlamydial infection. Chlamydial PAMPs recognition leads to an activation of the TLR signaling pathway which originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with a TIR domain-containing adaptor, MyD88. This association promotes the autophosphorylation of IL-1 receptor associate kinase (IRAK) by binding to MyD88. Activated IRAK will activate TRAF-6, which will stimulate the IKβ kinase complex (IKK). Activation and phosphorylation of IKK and its degradation leads to a translocation of NF-Kβ to the nucleus, promoting the expression of pro-inflammatory cytokines.

Intracellular nucleotide sensors play a role in innate response to *Chlamydia* as they induce the expression of type I interferons and other pro-inflammatory cytokines (Figure 3). Recent advances have focused upon the role of the receptors nucleotide-binding oligomerisation domain-containing 1 and 2 (NOD1 and NOD2) in chlamydial infection. NOD-like receptors (NLRs) participate in the initiation of innate responses against intracellular pathogens, recognizing common structural molecules of bacterial walls (e.g. peptidoglycan), and promote autophagy in infected cells (Travassos *et al.*, 2010). NOD1 is ubiquitous to most cell types of hematopoietic and non-hematopoietic origin, whereas NOD2 is mainly expressed by cell types of hematopoietic origin and some epithelial cell types. Both receptors and their downstream signaling pathways are necessary for the regulation of inflammatory responses and host cell defense against chlamydial infection (Zou *et al.*, 2016).

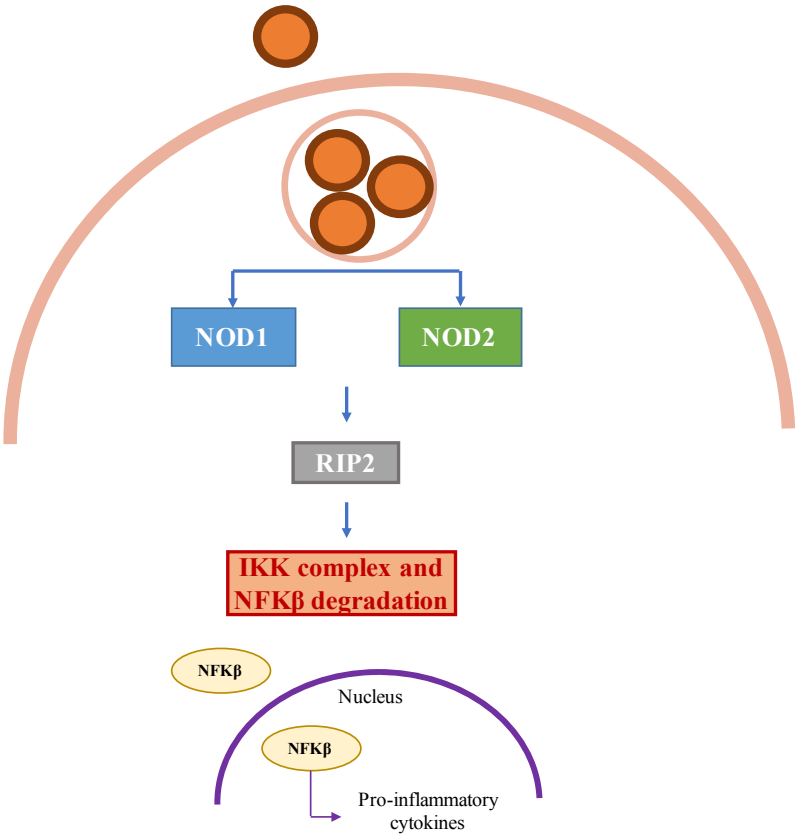


Figure 3. NOD-dependent inflammatory responses during chlamydial infection. After *Chlamydia* internalisation, cell wall molecules interact with NOD receptors, which will recruit the adaptor protein RIP2. RIP2 then mediates the activation of the IKK complex and MAPK pathway. The IKK complex can mediate the activation and nuclear translocation of NF-Kβ and the subsequent production of inflammatory chemokines, such as IL-8 and type I interferons.

1.1.2.3 Host innate adaptive response

The secretion of cytokines and chemokines by infected epithelial cells can recruit antigen-presenting cells (APC), such as macrophages and dendritic cells (DCs) to the site of infection. APCs maintain the inflammatory response producing more mediators as well as help the maturation of T cell, allowing the $CD4^+$ maturation into Th1 cells and its interaction with B cells. Activated B cells will produce specific antibodies directed against chlamydial proteins. Through antibody-dependent cellular cytotoxicity, $CD8^+$ T cells can lyse infected cells marked by antibodies attached to their surface, and opsonized elementary bodies are eliminated as antibody-antigen complexes through phagocytosis.

1.1.2.4 Persistence and immune response avoidance

Chlamydiae have evolved mechanisms to overcome the host immune response and persist inside the host. As an intracellular pathogen, its detection represents a challenge to the immune system. In its development, *Chlamydia* is confined within a membrane bound compartment protected from digestion, making the peptide recruitment for host MHC class II difficult. During stress, *Chlamydia* can enter a persistent state with low metabolic activity and replication. This state can lead to a decrease in pro-inflammatory cytokine production and consequent decline of inflammation intensity and other immune responses (Mpiga and Ravaoarinoro, 2006). Persistent chlamydial particles are hidden in host cells avoiding detection through humoral-mediated immunity. *Chlamydia* have also been shown to inhibit apoptosis in infected host cells, as well as having the capacity to manipulate several key steps of the host cell immune response. The chlamydial tail-specific protease (Tsp) can cleave NF-κB into two fragments, one of them retaining the ability to bind to DNA, but lacking the transactivation potential. Other antimicrobial mechanism that chlamydial infection can affect is the interferon production, including IFN-γ, which is associated with protection against viral, bacterial and protozoal infections. IFN-γ is linked to host cell tryptophan depletion, through activation and expression of the enzyme indoleamine 2-3 dyoxygenase (IDO) (Ziklo *et al.*, 2016). As some *Chlamydia* strains are tryptophan auxotroph, the inhibition of IFN-γ production and tryptophan depletion favour persistence and growth of these strains. To protect infected cells from detection by lymphocytes, Chlamydiae can inhibit IFN-γ-inducible MHC class expression and other mechanisms required for MHC gene activation (Zhong, 2009). These mechanisms therefore present a challenge for chlamydial detection and elimination by the immune system, leading to persistent infection.

1.1.2.5 Epidemiology

Chlamydia trachomatis is the most common cause of sexually transmitted bacterial infection worldwide with an estimated 130 million new cases annually (WHO). The large percentage of asymptomatic infections represents an ongoing source for efficient disease transmission. In the US, the Centers for Disease Control and Prevention studied the prevalence of genital *Chlamydia* in US population aged 14-39 years, between 2007-2012. The overall prevalence of *Chlamydia* among people aged 14–39 years was 1.7%, varying

by age and race/ethnicity. In women between 14-24 years, there was a higher prevalence of 4.7% (CDC, 2015). *C. trachomatis* infection is also a leading cause of pelvic inflammatory disease (PID) and female infertility, being attributed a 20-30% per cent of PID cases to this infection (Soper, 2010).

Blinding trachoma prevalence has also been a subject of extensive study. It is believed to be endemic in 56 countries and is the most prevalent in sub-Saharan Africa and East Africa. In the present day, trachoma has been reduced to developing countries as its prevalence has radically decreased in Europe and North America since the first half of the 20th century. It is currently estimated that 1.3 million people developed blindness due to *C. trachomatis* (Burton *et al.*, 2009) and it is still the most common cause of blindness caused by an infectious agent.

LGV infections had been uncommon in Western countries and its epidemiological data is very limited. In the recent years, some countries have experienced an increase in the number LGV diagnoses, being around 115% in Spain between 2009 to 2011 (Parra-Sánchez *et al.*, 2016) and 40% in the UK from 2014 to 2015 (Childs *et al.*, 2015). Human Immunodeficiency Virus (HIV)-positive men having sex with men (MSM) were the studied population with more LGV prevalence.

1.1.2.6 Treatment

The high percentage of asymptomatic infection, both in males and females, promotes chlamydial spread and the increase of clinical costs associated with this infection. Unfortunately, there is currently no specific effective vaccine for *Chlamydia* infection in humans (Igietseme, Eko and Black, 2011; de la Maza, Zhong and Brunham, 2017). The increased risk of transmission and co-infection with HIV, gonorrhea and HPV (Human Papilloma Virus) is an added cause for concern. Uncomplicated *Chlamydia* infection is normally treated with antibiotics. The standard anti-chlamydial treatments that are used are the macrolide azithromycin and the tetracycline doxycycline, with the macrolide erythromycin as the most used alternative treatment (Geisler, 2007)(Table 1). Tetracyclines are effective protein synthesis inhibitors that prevent the association of aminoacyl-tRNA with the bacterial ribosome, interfering with amino acid translocation

during translation and protein assembly by binding to a high-affinity binding site in the ribosomal 30S subunit. The absence of major anti-eukaryotic activity explains the selective antimicrobial properties of tetracyclines (Chopra and Roberts, 2001). Although mammalian cells have poor accumulation of these antibiotics and there is a relatively weak inhibition of protein synthesis supported by eukaryotic ribosomes, mitochondrial protein synthesis is affected (Moullan *et al.*, 2015). Azithromycin and the usual alternative antimicrobial treatment, erythromycin, shares the same mode of action as tetracyclines, bacterial protein synthesis inhibition, but by binding to the 50S ribosomal subunit. A meta-analysis of 12 randomized clinical trials of azithromycin versus doxycycline for the treatment of urogenital chlamydial infection demonstrated that the treatments were equally efficacious, with microbial cure rates of 97% and 98%, respectively (Geisler *et al.*, 2015). Erythromycin treatment is often associated with gastrointestinal side effects that can lead to non-adherence with treatment, which makes it less ideal than tetracycline treatment (Workowski, Bolan and Centers for Disease Control and Prevention, 2015). The described treatments are currently effective as no antibiotic resistance has yet been reported in human *Chlamydia trachomatis* infection.

Table 1. Recommended treatment regimens and alternative regimens for *C. trachomatis* infection in non-pregnant patients. (Workowski, Bolan and Centers for Disease Control and Prevention, 2015).

Recommended regimens	Alternative regimens
Azithromycin 1g orally in a single dose	Erythromycin base 500mg or Erythromycin ethylsuccinate 800mg orally 4 times a day for 7 days
Doxycycline 100mg orally twice a day for 7 days	Levofloxacin 500mg orally once daily for 7 days
	Ofloxacin 300mg orally twice a day for 7 days

1.1.3 Waddlia chondrophila

Waddlia chondrophila is a *Chlamydia*-like intracellular bacteria, which shares a biphasic life cycle with the other chlamydial species. *Waddlia* is a genus of bacteria within its own family, which belongs to the Chlamydiae phylum. *Waddlia chondrophila* was first isolated from tissues of aborted bovine foetus at the Washington Animal Disease Diagnostic Laboratory by Dilbeck *et al.* in 1986. When it was first studied by light microscopy, it was clear that the microorganism was multiplying inside a cytoplasmic vacuole in the host cell, exhibiting structural characteristics compatible with those of Rickettsiae and Chlamydiae. Serological identification with monoclonal and polyclonal antisera to a variety of *Rickettsia*, *Coxiella*, *Wolbachia*, *Anaplasma* or *Chlamydia* spp. (Dilbeck *et al.*, 1990) failed to help taxonomical classification. Genomic studies were then carried out focusing on the 16S ribosomal DNA gene sequences (Rurangirwa *et al.*, 1999). 16S rDNA sequence analysis indicated that *Waddlia chondrophila* was closely related to the members of the order Chlamydiales, having 84.5-85.3% sequence similarity. This degree of similarity was high enough to allow *W. chondrophila*'s inclusion in the order Chlamydiales but not to justify its inclusion in the Chlamydiaceae family (Everett and Andersen, 1997; Everett, Bush and Andersen, 1999). *Waddlia chondrophila* has shown biochemical and antigenic differences to other families of the phylum. Contrary to Chlamydiceae species, *W. chondrophila* is resistant to penicillin. *W. chondrophila* and other *Chlamydia*-like organisms exhibited better survival rates in host-free conditions or under heat exposure compared to *C. trachomatis* (Coulon *et al.*, 2012). Other difference between *Waddlia chondrophila* and chlamydial development in the host cell is the level of cytoskeletal arrangement modification in the host cell during infection. *C. trachomatis* inclusions are tightly embedded in cytoskeletal elements, whereas in *Waddlia*, cytoskeleton arrangement around the inclusions were less pronounced (Dille *et al.*, 2015). Contrarily to other Chlamydiales, *W. chondrophila* evades the endocytic pathway and co-localize with the mitochondria and endoplasmic reticulum. *Waddlia chondrophila* does not depend on Golgi apparatus fragmentation during infection, although this process is important for chlamydial replication (Heuer *et al.*, 2009; Dille *et al.*, 2015). *Waddlia* recruits the mitochondria to a much larger extent than other *Chlamydia* species, allowing the access to large quantities of lipids and ATP. Genetic analysis has revealed higher biosynthetic abilities for essential compounds such as

nucleotides and amino acid synthesis in *Waddlia chondrophila* than in other *Chlamydia* species, suggesting less host dependency.

1.1.3.1 Clinical manifestations

The natural host of *Waddlia chondrophila* is not known. After the isolation of *Waddlia chondrophila* from aborted bovine foetuses, several studies were carried out to determine the association between *Waddlia chondrophila* and abortion. Baud *et al.* performed a study between 2006 and 2009 on women given a diagnosis of an acute episode of miscarriage and women having uneventful pregnancies without previous history of miscarriage, stillbirth or preterm labour. The study confirmed an association between miscarriage and the presence of *W. chondrophila* (Baud *et al.*, 2014). Identification of *W. chondrophila* in the human genital region pointed to a sexual transmission and infection. A serological study based on the presence of sexually transmitted *Chlamydia trachomatis* and *Waddlia chondrophila* was performed (Baud *et al.*, 2007) to identify any correlation that would confirm the possible sexual transmission of *W. chondrophila*. The absence of this correlation suggested that *W. chondrophila* is not sexually transmitted. However, DNA from this *Chlamydia*-like organisms has also been found in respiratory samples from patients with community-acquired pneumonia suggesting a possible role in human respiratory disease (Haider *et al.*, 2008). Some studies have also shown an association between contact with animals and positive serologic results for *Waddlia*, raising its zoonotic potential (Corsaro and Greub, 2006; Baud *et al.*, 2007). Furthermore, standing well water could also be an important potential reservoir and a possible infection source of *W. chondrophila* (Codony *et al.*, 2012).

1.1.3.2 Host cell response

As with other members of the Chlamydiales order, attachment and entry in the host cell is essential for survival and replication. Little is known about the host cell response to infection and about the molecules implicated in the adhesion of *Waddlia chondrophila* to its host cell. In *Chlamydia*, several bacterial proteins have been identified in adhesion, such as the major outer membrane protein (MOMP) and OmcB. A study of the genome of *Waddlia chondrophila* revealed the existence of a novel outer membrane protein

(OMP) family comprising 11 putative β-barrel proteins or porins (Bertelli *et al.*, 2010). The presence of a modified OmcB gene and HSP60 gene was also found, pointing to similar entry interactions with *Chlamydia spp. W. chondrophila* also possess a Type III secretion system, sharing almost all its encoding genes with other *Chlamydia* species. Bertelli *et al.,* found that the effectors injected by Waddlia T3SS were different from those injected by other species in the Chlamydiaceae family. Only one effector was found that was common to both *Chlamydia* species and *Waddlia*, Pkn5. The innate immune response to infection to *W. chondrophila* infection was established in the ovine trophoblast cell line AH-1, showing a high similarity to the one obtained after infection with *Chlamydia abortus* (Wheelhouse *et al.*, 2009). An induction of the expression of IL-8 and TNF-α as a result of *W. chondrophila* infection was found (Wheelhouse *et al.*, 2014). It was also shown that IL-8 production was dependent on active infection and intracellular invasion, as UV-killed *W. chondrophila* failed to induce IL-8 production in AH-1 cells. The decrease in IL-8 production was also found in UV-killed *C. trachomatis* L2 cells, which was shown to be a result of the intracellular association between TLR2 and the chlamydial inclusion (O'Connell *et al.*, 2006). The lack of IL-8 production after UV-killed *C. trachomatis* and *W. chondrophila* exposure suggest that host cell response against chlamydial infection is primarily triggered by intracellular receptors, such as NOD1, rather than by cell surface receptors. Other receptors have been proposed to elicit IL-8 release after PRR recognition, as IL-8 production was also found in *W. chondrophila* infected wild-type HEK293 cells, which do not express TLR2 or NOD2, and NOD1 only at very low levels (Storrie *et al.*, 2016). In addition, the proinflammatory cytokine IL-1β was found to be produced by host cells exposed to UV-killed *W. chondrophila* indicating the stimulation of different immune pathways by the pathogen. More studies still need to be performed to compare mechanisms underlying the innate and adaptive immune response to *Waddlia chondrophila* and *Chlamydia spp*.

1.1.3.3 Persistence and immune response avoidance

Persistence in the host cell under stress situations has been observed in *W. chondrophila* infection (Vasilevsky *et al.*, 2015), although it is expected to be maintained through differential pathways than in *Chlamydia* infection. The growth of *W. chondrophila* in different host cell lines, such as the endometrial Ishikawa cells, was studied (Kebbi-

Beghdadi, Cisse and Greub, 2011). A decrease in the pathogen replication and the spontaneous appearance of large aberrant bodies was seen in Ishikawa cells, both processes being often associated to persistence. After internalization, *W. chondrophila* could avoid the host endocytic pathway as occurs with the other members of the Chlamydiaceae family. *W. chondrophila* was found to be more virulent than *C. trachomatis* as it was less host dependent. Some characteristic features of *Chlamydia* infection and persistence, such as inhibition of host cell apoptosis, are also not observed during *W. chondrophila* infection.

1.1.3.4 Epidemiology

Approximately 15% of pregnancies end in miscarriage, although the cause is identified in only 50% of these cases. *Waddlia chondrophila* infection has been associated with human miscarriage. Seroprevalence of *W. chondrophila* was higher for women who had sporadic (31.9%) and recurrent (33.0%) miscarriages than for women who had uneventful pregnancies (Baud *et al.*, 2007, 2014)(Baud *et al.*, 2014). In 2015, a study investigating seroprevalence of *W. chondrophila* in subfertile women was performed by Verweij *et al.* (Verweij *et al.*, 2015). A higher seroprevalence of 45.5% was found in this instance and therefore, more studies need to be performed on a larger scale to determine the prevalence and morbidity of miscarriages caused by this pathogen. The role of *Waddlia chondrophila* in miscarriage is not fully understood and thus there are no established screening programs to evaluate the risk of having a miscarriage due to the presence of *Waddlia chondrophila*.

1.1.3.5 Treatment

Waddlia chondrophila infection is normally only detected after infertility has been diagnosed or a miscarriage has occurred. As a result, there is no established treatment for *W. chondrophila* infection. Previous research has reported *W. chondrophila* resistance to penicillin, in contrast to many other *Chlamydia* species. Goy *et al.,* tested the antibiotic susceptibility of *W. chondrophila in vitro* and found it was susceptible to the most commonly used treatments for *Chlamydia* infection, azithromycin and doxycycline (Goy and Greub, 2009). In contrast, the pathogen exhibited resistance to quinolones and β-

lactams. Azithromycin is therefore a potentially effective treatment for *Waddlia chondrophila* infections due to its proven efficacy against other members of the order Chlamydiales. However, as *W. chondrophila* has been found after unsuccessful pregnancies, doxycycline is unlikely to be used pre-birth, given its contraindications in pregnant patients. What is clear is that more research needs to be performed to establish the activity of these antibiotics against *W. chondrophila in vivo*.

1.2 Antimicrobial resistance

Antimicrobial resistance is the ability of microbes to resist the effects of drugs. This resistance allows the pathogen to survive and continue its growth. Antimicrobial resistance is a global concern as it directly results in increased morbidity, mortality and costs of health care. Antibiotics are drugs predominantly used to prevent and treat bacterial infections. When the bacteria develop mechanisms to resist the treatment, antibiotic resistance occurs, resulting in a harder to treat infection. Antibiotic resistance is a natural process of bacterial evolution, which can be slowed with prevention and the appropriate use of antibiotics, but not stopped. Therefore, there is an urgent necessity to find new antimicrobial drugs, with low toxicity and a low likelihood of engendering antimicrobial resistance.

1.2.1 Antibiotic resistance in Chlamydia species

There are relatively few documented reports of antibiotic resistance in *Chlamydia* and no examples of natural and stable antibiotic resistance in strains collected from humans (Sandoz and Rockey, 2010). Several clinical isolates have shown resistance to antibiotics but these strains either lost their resistant phenotype *in vitro* or their viability. Antibiotic resistance has not emerged yet in human chlamydial infection although a stable tetracycline-resistance phenotype has been described in *Chlamydia suis* isolates from ill and apparently healthy pigs (Donati *et al.*, 2016). The possibility of horizontal transfer of tetracycline-resistance from *C. suis* to clinical strains of *Chlamydia trachomatis in vitro* has been object of study (Suchland *et al.*, 2009). Horizontal transfer of tetracycline-resistance was observed, pointing to a future possibility of it in a naturally occurring *C. trachomatis* strain. Although antibiotic resistance in *Chlamydia trachomatis* is still rare,

there has been considerable recent concern about the efficacy of treatments for urogenital and anorectal forms, and the efficacy of the most frequent treatment, azithromycin. Recent meta-analysis of treatment efficacy for urogenital *C. trachomatis* infection between azithromycin and doxycycline revealed a significant efficacy difference in favour of doxycycline (Kong and Hocking, 2015). Another meta-analysis was performed in 2015 in instances of anorectal infections revealing a higher reduction in the efficacy of azithromycin (efficacy of 83%, with 95% efficacy being the threshold recommended by the World Health Organisation, WHO for STI treatments). Alternatives are needed as there is not a vaccine available to prevent *Chlamydia* infection in humans and the most used treatment may be subject of resistance in the future.

1.3 Cationic Host Defence Peptides

Host defence peptides (HDPs; also known as antimicrobial peptides), are peptides that have broad antimicrobial and immunomodulatory activity. They are key components of the innate immune system, with potent microbicidal, antiviral and immunomodulatory properties (Hemshekhar, Anaparti and Mookherjee, 2016). HPDs can be found in a variety of life-forms, including prokaryotes and eukaryotes. The first reported animal-originated HDP is a defensin, which was isolated from rabbit leukocytes in 1956 (HIRSCH, 1956). In total, more than 2,600 HDPs have been discovered or characterised up to date. In animals, HDPs are normally produced in tissues and organs that are exposed to pathogens. They are believed to be the first line of defence in the innate immune response against bacteria, viruses and protozoa. Although there is great sequence diversity among HDPs, there are structural elements that are shared by most of them. They are small amphipathic peptides, typically 12-50 amino acids in length, with a net positive charge ranging from +2 to +9 due to an abundance of basic amino acids such as lysine and arginine (Hemshekhar, Anaparti and Mookherjee, 2016). HDPs present different secondary structures although two forms are predominant; β-sheet structures with disulphide bonds and α-helical structures adopted by linear peptides. A feature common to all cationic host defence peptides is that they exert their activity by targeting membranes (Mookherjee and Hancock, 2007). The two most well characterized families of CHDPs in mammals are cathelicidins and defensins.

1.3.1 Defensins

This family of peptides, originally isolated from leukocytes, is expressed in immune and epithelial cells. Defensins are produced by vertebrates and have been also found in plants and insects. A common feature of defensins is the three-stranded β-sheet structure, with 3 disulphide bridges formed between their 6 cysteine residues. Defensins are categorized into two main subfamilies, the α and β defensins, which differ in the length of peptide segments between the six cysteines and in the pairing of the cysteines that are connected by disulphide bonds. A third subfamily has been discovered, the θ defensins. θ defensins have a cyclic structure formed from two α defensins. Usually, newly synthesized defensins undergo proteolytic cleavage to become active. Most active defensins show antimicrobial activity against bacteria and fungi, achieved by their ability to permeabilize microbial membranes (Ganz, 2003).

1.3.2 Cathelicidins

Cathelicidins are a family of evolutionary conserved peptides found in a variety of species. The discovery of cathelicidins commenced after the isolation of the antimicrobial peptide Bac5 from bovine neutrophils, and the finding that they were cleaved from inactive precursors (Gennaro, Skerlavaj and Romeo, 1989). Many cathelicidin peptides have been described in a substantial variety of animals such as lizards, birds, fish and mammals. Cathelicidins range in size, from 12 to 88 amino acids, and are comprised of a highly conserved 14 kDa N-terminal cathelin-like pro-domain, followed by a signal peptide and a C-terminal 'mature' peptide region (Figure 4). The cathelin-like domain is highly conserved among species and shares homology with the porcin cathepsin cysteine protease inhibitor, the term 'cathelin' standing then for *cathep*sin *L* inhibitor. The C-terminal domain is highly divergent and is responsible for the antimicrobial activity of the peptide upon proteolytic cleavage. The signal peptide assures the direction of newly synthesized peptides towards the secretory pathway. In humans, only one cathelicidin has been described, the peptide hCAP18, which is encoded by the cathelicidin antimicrobial peptide (CAMP) gene, and is cleaved to the active peptide LL-37.

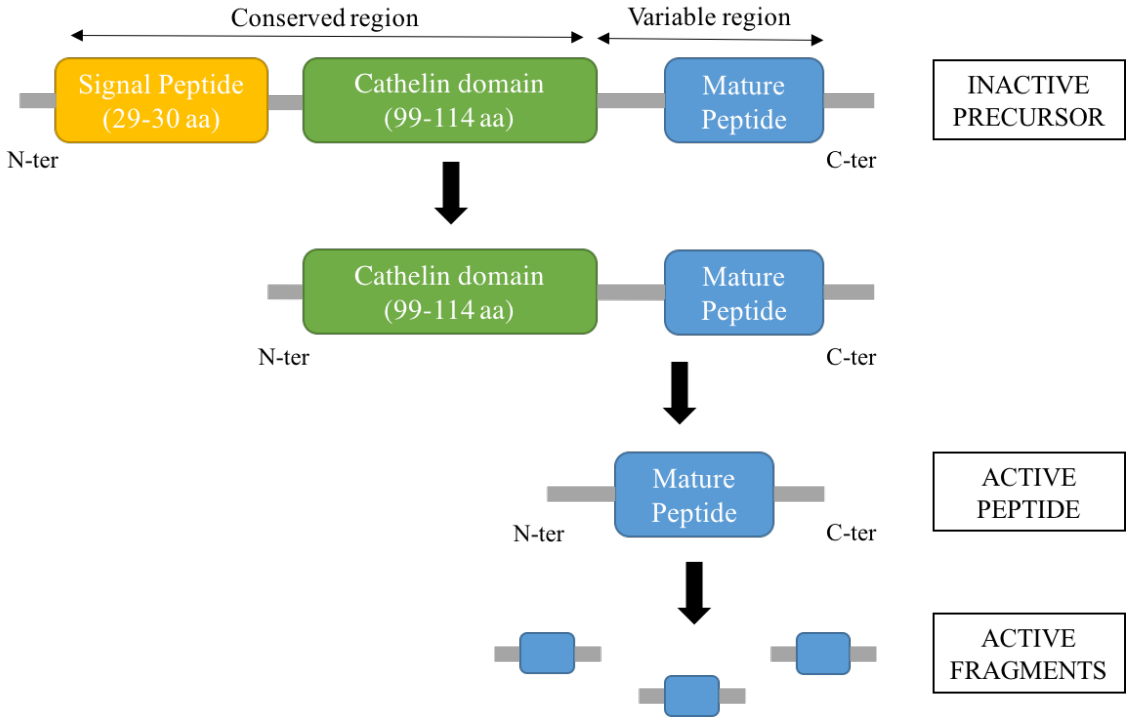


Figure 4. Structure and processing of cathelicidin peptides. Adapted from Shinnar *et al.*, (Shinnar, Butler and Park, 2003). Cathelicidins are expressed and synthesized as inactive pro-peptides. A signal peptidase removes the N-terminal signal peptide. The mature peptide is released by further proteolytic processing of the precursor by different processing enzymes, removing the cathelin domain. The mature peptide can be cleaved into active fragments.

1.3.2.1 hCAP18 (LL-37)

hCAP18 is encoded by the CAMP gene, which is found on the chromosome 3p21.3 (Larrick *et al.*, 1996). hCAP-18 is a major protein in specific granules of neutrophils, but it is also present in subpopulations of lymphocytes and monocytes, in squamous epithelia, epididymis and seminal plasma, in the lung and in keratinocytes during inflammatory skin diseases (Sørensen *et al.*, 2001). hCAP18 is cleaved extracellularly by proteinase-3, a serine protease, to generate LL-37 which is the dominant cleavage product. LL-37 structure is linear and composed by 37 amino acids with two leucine residues at the N-terminal which adopts an amphipathic α-helix structure (Figure 5). Other cell types of the immune system that can express LL-37 are macrophages, eosinophils, mast cells, Natural Killer, T and B cells (Dürr, Sudheendra and Ramamoorthy, 2006). hCAP-18 can be found

in body fluids such as saliva, sweat, blood, semen, milk and airway surface fluids. Physiological LL-37 concentration in plasma is approximately 1.2µg/ml (Sørensen *et al.*, 1997), though this concentration can increase in response to infection to concentrations up to 20µg/ml in the airway fluid of infected patients (Barlow *et al.*, 2014).

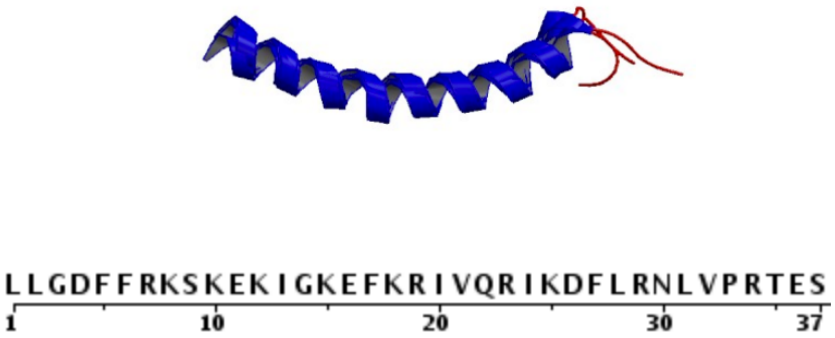


Figure 5. Amino acid sequence and secondary structure of LL-37. PDB ID: 2K6O (Wang, 2008). LL-37 secondary structure was obtained by NMR spectroscopy. The three-dimensional structure is composed of a curved amphipathic α-helix structure.

1.3.2.2 Protegrin-1

Contrary to humans, other mammals can produce more than one cathelicidin. In the case of pigs, they express a variety of cathelicidins which differ in activity and structural motifs. The porcine cathelicidins include the protegrins (PGs), the α-helical peptides (PMAPs), prophenins and the PR-39 peptide. Antimicrobial activity has been well characterized for PR-39 (Agerberth *et al.*, 1991), PMAP-23 (Zanetti *et al.*, 1994), PMAP-36 (Scocchi *et al.*, 2005), and PG-1 (Kokryakov *et al.*, 1993). There are five small protegrins (16-18 amino acids residues) described (Kościuczuk *et al.*, 2012). As cathelicidins, protegrins are proteolytically cleaved into their active forms by neutrophil elastase in the extracellular environment. Protegrins present a two-stranded β-sheet structure in solution which is linked by a β-hairpin loop stabilized by to intramolecular disulfide bonds between cysteines (Figure 6). Protegrin peptides interact strongly with lipid bilayer membranes, particularly anionic lipids; protegrins have been shown to form pores in lipid bilayers, which can lead to the pathogen death (Bolintineanu and Kaznessis,

2011). Protegrins have antimicrobial activity against bacteria, especially Gram-negative, fungi, and some enveloped viruses (Sousa *et al.*, 2017). Protegrin-1 is able to retain antimicrobial and antifungal properties at physiologic salt concentrations and in the presence of serum, conditions in which other cathelicidins would be affected (Steinstraesser *et al.*, 2001).

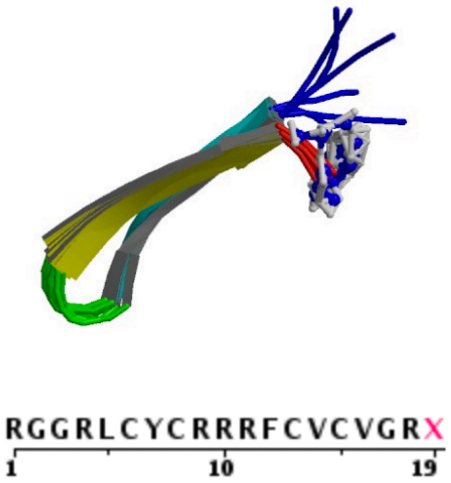


Figure 6. Amino acid sequence and secondary structure of PG-1. PDB ID: 1PG1 (Fahrner *et al.*, 1996). PG-1 secondary structure was obtained by NMR spectroscopy. Protegrin-1 form a structure composed primarily of a two-stranded antiparallel β-sheet, with strands connected by a β-hairpin loop. The red 'X' at the end of the peptide sequence represents an unspecified or unknown amino acid.

1.3.2.3 SMAP-29

In sheep eight cathelin-associated peptides have been identified, including cyclic dodecapeptide, SMAP-28 and 29 and Bac5, 6, and 7.5. The cathelicidin SMAP-29, sheep myeloid antimicrobial peptide of 29 residues, possesses an α-helical structure (Figure 7) and is one of the most effective HDP known. This peptide possesses broad spectrum antimicrobial activity, against both bacteria and fungi. SMAP-29 is produced by several cell types, including trophoblasts (Coyle *et al.*, 2016). Dawson *et al.,* studied the antimicrobial potential of SMAP-29 against several bacterial and fungal species (Dawson and Liu, 2009). They discovered that this peptide inhibits the growth of bacterial pathogens, Gram – and Gram +, at a very low MIC (minimum inhibitory concentration,

the lowest peptide concentration that prevents growth), proving its potent antimicrobial activity. When they were treating *Bacillus anthracis* infection, the obtained MIC was 0.8-1.6μM. Very few antimicrobial peptides are active at concentrations lower than this (Dawson and Liu, 2008). As with other antimicrobial peptides, high concentrations can produce cell cytotoxicity, probably due to its capacity to disrupts membranes. SMAP-29 is not active in the presence of serum.

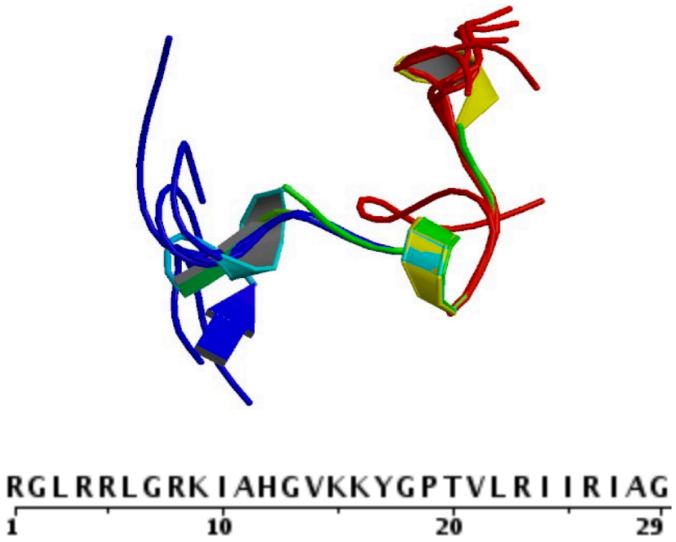


Figure 7. Amino acid sequence and secondary structure of SMAP-29. PDB ID: 1FRY (Tack *et al.*, 2002). SMAP-29 secondary structure was obtained by NMR spectroscopy. The three-dimensional structure indicated that residues 8-17 were helical, residues 18-19 formed a hinge and residues 20-28 formed and hydrophobic segment.

1.3.2.4 Immunomodulatory functions of cathelicidins

Cathelicidins have multiple functions in immunity and inflammation. Some peptides such as the human cathelicidin LL-37 have been proposed to have far more potent immunomodulatory activities than antimicrobial functions (Turner *et al.*, 1998). It is believed that their broad antimicrobial properties are largely attributed to their capacity to mediate the immunomodulatory functions. HDPs constitute a link between innate defense and adaptive immunity due to their chemotactic activity. Cathelicidins can promote chemotaxis of immune cells in an indirect manner by inducing the production of

chemokines from immune and epithelial cells. For example, cathelicidins can stimulate epithelial cells to release IL-6, as well as cause mast cell degranulation, promoting histamine and other cytokines release. LL-37 upregulates inflammatory signaling due to its ability to activate cell receptors, such as TLRs (Vandamme *et al.*, 2012). Besides chemotaxis, LL-37 has been also found to suppress neutrophil apoptosis, prolonging their survival, and potentially protecting the host against the pathogen for a longer period (Barlow *et al.*, 2006; Li *et al.*, 2009). The human cathelicidin has also the ability to induce dendritic cell differentiation (Yang, Chertov and Oppenheim, 2001) and Th1 cytokine production.

1.3.2.5 Antibacterial activity of cathelicidins

Cathelicidins have shown direct antimicrobial activity. The importance of their broad antimicrobial activity, against virus, bacteria and fungi is evidenced through an increased susceptibility to infection in neutropenic patients, who have less neutrophils and consequently a decrease in the cathelicidin concentration (Neth *et al.*, 2005). More evidence of the antibacterial activity of cathelicidins is found in mice deficient in mCRAMP (mouse cathelicidin) which have an increased susceptibility to infection of the skin, urinary tract and gastrointestinal tracts (Iimura *et al.*, 2005; Chromek *et al.*, 2006). Experimental systems simulating physiologically conditions and *in vivo* animal model infections have demonstrated how CHDP are able to limit or clear infections (Barlow *et al.*, 2010). All cathelicidins are positively charged and amphipathic. The positive charge allows them to interact with negatively charged phospholipids in target membranes. Their partial hydrophobicity is important for its subsequent insertion into the membrane (Figure 8). The peptide insertion in the membrane leads to the formation of membrane pores that cause an unrestricted flux of ion and other molecules from the cytoplasm (Dawson and Liu, 2009; Bolintineanu and Kaznessis, 2011). The antiviral activity of cathelicidins has been also studied, although not as broadly as in bacteria, suggesting that cathelicidins may prove to play significant roles in defense against viral infections (Barlow *et al.*, 2014).

Figure 8. Example of cathelicidin (PG-1) pore formation in a phospholipidic membrane. (Bolintineanu and Kaznessis, 2011). After adsorption to the surface of anionic bacterial membranes, the peptide inserts into the membrane core. Oligomerisation of peptides to form aggregates leads to transmembrane pore that will cause uncontrolled ion and contents leakage leading to bacterial death.

1.3.2.6 Antibacterial activity of cathelicidins in Chlamydia infection

The effect of different cathelicidins on *Chlamydia* has been previously studied. The porcine CHDP, Protegrin-1, was found to have strong anti-chlamydial activity against *C. trachomatis* (Chong-Cerrillo *et al.*, 2003) inhibiting infectivity by 50% at a concentration of 6μg/ml. The antimicrobial activity of other cathelicidin peptides (SMAP-29 from sheep, LL-37 from humans, BMAP-27 and BMAP-28 from cattle apart from Protegrin-1) was tested against a total of 25 strains from the chlamydial species (Donati *et al.*, 2005). It was observed than *C. trachomatis* was more susceptible to peptides than *C. pneumoniae* strains. SMAP-29 was found to be the most active peptide among various chlamydial species, including *C. trachomatis* and *C. pneumoniae.* Donati *et al*. also tested the same cathelicidins against *Simkania negevensis*, Gram-negative bacteria belonging to the family of Simkaniaceae in the order Chlamydiales. *S. negevensis* was not sensitive to LL-37, even at very high concentrations of the peptide (80 μg/ml). On the contrary it was found to be susceptible to the other tested peptides at very low concentrations (1 to 0.1μg/ml). These results suggested a higher susceptibility of *Simkania negevensis* than other members of Chlamydiales (Donati *et al.*, 2011). However, to date, no studies have been performed to study the antimicrobial effects of cathelicidins in *Waddlia chondrophila* infection.

1.4 Hypothesis

Cathelicidins have shown a broad antibacterial spectrum and effectiveness against different bacteria and have been previously study in *Chlamydia* infection with promising results. *In vitro*, different *Chlamydia* species have been shown to be susceptible to cathelicidins (Yasin *et al.*, 1996; Chong-Cerrillo *et al.*, 2003; Donati *et al.*, 2005, 2010). The direct effect of HDPs has been the most studied. Their indirect antimicrobial effects have been recognized, in addition to direct microbicidal activity, and are believed to be key in the control of inflammation (Yang *et al.*, 2004).

Waddlia chondrophila has been proposed as possible model for *Chlamydia* infection thanks to its faster life cycle and its higher virulence. We hypothesise that direct cathelicidin exposure would have an impact on *Chlamydia trachomatis* and *Waddlia chondrophila* population, decreasing its replication and infectivity. This study will also focus on the effects of cathelicidin treatment in inflammation and host cell protection against infection, which we expect to be modulated due to the broad immunomodulatory properties of cathelicidins. The effect of the different cathelicidin treatment on host cell death will also be study to assess their toxicity.

1.5 Research Aims

I. To assess the direct effect of LL-37, PG-1 and SMAP-29 on *C. trachomatis* and *W. chondrophila* infectivity and replication.

II. To study the indirect effect of LL-37, PG-1 and SMAP-29 on *C. trachomatis* and *W. chondrophila* infectivity and replication.

III. To study the effect of the different cathelicidin treatments on host cell death.

IV. To investigate the host inflammatory response after the different cathelicin treatment on *C. trachomatis* and *W. chondrophila* infection.

1.6 Research Objectives

I. To achieve aim I, we will use *in vitro* models of *C. trachomatis* and *W. chondrophila* infection and subject both the pathogens to varying concentrations of peptides prior to host cell infection. Bacterial concentrations and infectivity will then be assessed by molecular quantification (DNA quantification) and by immunostaining of intracellular inclusions respectively.

II. To achieve aim II, *in vitro* models of *C. trachomatis* and *W. chondrophila* infection will be used. The host cells will be exposed to varying concentrations of peptides prior to infection. Bacterial concentrations and infectivity will be assessed by quantitative PCR and immunostaining.

III. To accomplish aim III, we will use the same *in vitro* epithelial host cell model as in aim I and II. Bacteria will be exposed to varying concentrations of peptides prior to host cell infection. Afterwards, the bacteria and the peptides will be added to the host cell monolayers and incubated. To assess if the cathelicidin treatments caused host cell death, supernatants will be recovered and analysed by lactate dehydrogenase cell death assay.

IV. To achieve aim IV, we will use *in vitro* models of *C. trachomatis* and *W. chondrophila* infection and subject both the pathogens to varying concentrations of peptides prior to host cell infection. In parallel, the host cells will be also exposed to the peptides prior to infection with both pathogens. Supernatants will be recovered and analysed by Enzyme Linked ImmunoSorbent Assay to investigate the host cell inflammatory response.

2 Materials and methods

2.1 Cell culture

The human epithelial cell line, HEp2, was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). HEp2 were routinely grown in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Paisley, UK) supplemented with 5% heat inactivated foetal bovine serum (FBS, Life Technologies, Paisley, UK) and 0.1% Gentamycin (10mg/dL) (Life Technologies, Paisley, UK). Cells were grown at 37^{o}C in a heated, humidified incubator with 5% CO_2. Cells were passaged every 3-4 days and cell counts were performed using a hemocytometer to determine seeding density.

2.2 Cathelicidins

Cationic Host Defence Peptides (CHDP) were provided through a collaborative agreement with the Biotechnology Core Facility at the US Centers for Disease Control & Prevention (Atlanta, GA, USA). The peptides were assembled using the Fmoc/tBu solid-phase peptide synthesis approach (Zughaier *et al.*, 2010) using either model 433A (Applied Biosystems, CA, USA) or model Liberty (CEM Corporation, NC, USA) automated peptide synthesizers followed by cleavage in the trifluoroacetic acid (TFA) / phenol / thioanisole / ethanedithiol/water (10∶0.75∶0.5∶0.25∶0.5, w/w) mixture at 25°C for 90 minutes followed by precipitation with cold diethyl ether. The crude peptides were purified by preparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The peptide purity (>98%) was confirmed by analytical RP-HPLC, and the masses were confirmed by mass spectrometry. Following lyophilisation, the purified peptides were obtained in the form of their TFA salts. The peptides used for the experiments were the human cathelicidin LL-37 peptide (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), scrambled LL-37 (sequence RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL), porcine cathelicidin Protegrin-1 (sequence RGGRLCYCRRRFCVCVGRX) and the sheep cathelicidin SMAP-29 (sequence RGLRRLGRKIAHGVKKYGPTVLRIIRIAG). All the peptides

were dissolved in endotoxin-free water (Sigma-Aldrich, Dorset, UK) and stored at -20^{o}C until use.

2.3 Chlamydia trachomatis and Waddlia chondrophila propagation

Waddlia chondrophila strain ATCC VR-1470 was grown at 37°C in HEp2 cells, with Infectious Media (IM) which consisted of IMDM supplemented with 2% heat inactivated FBS and cycloheximide (1μg/ml final concentration). After 72 hours, the cell monolayers were disrupted with cell scrapers and the medium containing cell debris was removed, before centrifugation at 50×g for 5 minutes at 4°C to remove intact cells. The supernatant was removed and centrifuged 10 minutes at 20,000×g. The pellet was resuspended in ice-cold sucrose-phosphate-glutamic acid (SPG) buffer (10mM, pH 7.4), aliquoted into microcentrifuge tubes and stored at −80°C. To quantify viable organisms, aliquots were thawed at room temperature and titrated on HEp2 cells.

Chlamydia trachomatis serovar LGV II strain ATCC VR-902B was grown at 37°C in HEp2 cells, with IMDM supplemented with 2% heat inactivated FBS and cycloheximide (1μg/ml final concentration). After 72 hours, cell monolayers were disrupted with cell scrapers and the medium containing the cell debris was centrifuged at 2851xg for 10 minutes to pellet the infected cells. The supernatant was discarded and the pellet was resuspended in 1-2ml 10% ice cold PBS. Glass beads were added to the solution and it was then vortexed for 1 minute. The solution was centrifuged at 233xg for 5 minutes and the supernatant was aliquoted in microcentrifuge tubes adding an equal amount of filtered 4SP buffer (Sucrose 0.67mM; Na_2HPO_4 80.4μM, pH 7.1) and stored at -80ºC.

2.4 Determination of bacterial titer

HEp2 cells were seeded at a density of $5x10^4$ cells per chamber in 8 well chamber slides (BD Falcon, Becton Dickinson, Bedford, UK) and grown to 80-90% confluency. Serial dilutions (10-fold) of the obtained aliquots from the bacterial propagation were prepared from a -2 to a -8 dilution factor in IM. Culture media was aspirated before addition of 250μl of each serial dilution to each chamber. Cells were incubated with the bacteria for 1 hour at 37ºC. Infectious supernatant was aspirated and replaced with fresh culture

media. After 24 hours the medium was removed, cells were fixed in acetone, air-dried, and the slides were frozen at −20°C prior to analysis by fluorescent immunocytochemistry. To obtain the number of bacteria per ml of the stock aliquots, inclusions were counted in two chambers corresponding to two different serial dilutions with a countable average number bacterial inclusions (20~200). The average number obtained was then transformed using the dilution factor used and then multiplied by four to obtain the final concentration of bacteria per ml of the stock aliquots.

2.5 Direct cathelicidin treatment of C. trachomatis and W. chondrophila

HEp2 cells were seeded at a density of $5x10^4$ cells per chamber in 8 well chamber slides (intracellular bacterial inclusions immunostaining assays) or at a density of $2x10^5$ per well in 24 well plate (bacterial quantification through real-time PCR assays) and grown to 80-90% confluency.

Waddlia chondrophila was diluted to a Multiplicity of Infection (MOI) of 1 and *C. trachomatis* was diluted to a Multiplicity of infection (MOI) of 0.01 in serum-free IMDM media and incubated for 1 h at 37ºC with various concentrations of LL-37, PG-1 and SMAP-29: 0µg/ml, 10µg/ml, 25µg/ml, 50µg/ml and 100µg/ml. A 1:10 dilution of these concentrations was prepared in 2% FBS supplemented IMDM was carried out prior addition to cells for a further 1 h incubation at 37ºC. Media with bacteria was then aspirated and replaced with 5% FBS supplemented IMDM for 24 hours at 37ºC. Following the last incubation, monolayers grown in the 24-well plate were chemically lysed and DNA extraction was performed. Immunostaining was performed in parallel monolayers grown in chamber slides. Supernatants were recovered and stored at -80ºC. LL-37, PG-1 and SMAP-29 treatments were performed independently.

2.6 Host cell exposure to cathelicidins prior infection

HEp2 cells were seeded at a density of $5x10^4$ cells per chamber in 8 well chamber slides (intracellular bacterial inclusions immunostaining assays) or at a density of $2x10^5$ per well in 24 well plate (bacterial quantification through quantitative PCR, qPCR, assays) and grown to 80-90% confluency.

LL-37, PG-1 and SMAP-29 were diluted in serum-free IMDM to various concentrations (0µg/ml, 1µg/ml, 5µg/ml and 10µg/ml) and incubated for 1 hour at 37ºC with the HEp2 host cells. LL-37, PG-1 and SMAP-29 treatments were performed independently. Host cell monolayers were then washed once with 1X PBS and then infected at identical MOIs than when they were infected with cathelicidin treated bacteria (Results Part 2.6) for 1 hour at 37ºC. After infection, media with bacteria was discarded and replaced with 5% FBS supplemented IMDM, and cells were incubated for 24 hours at 37ºC. Following the last incubation, monolayers grown in 24-well plate were lysed and DNA extraction was performed. Immunostaining was performed in parallel in monolayers grown in chamber slides.

2.7 DNA extraction

DNA was extracted using an DNeasy® Blood and Tissue Kit (Qiagen, Crawley, UK) according to manufacturer's protocol. Cell monolayers used for quantitative PCR analysis were lysed directly in 200µl AL Lysis buffer (supplied with DNeasy Blood and Tissue kit). The lysate was mixed sequentially with 200µl PBS and 20µl Proteinase K before incubation at 56°C for 30 minutes. Absolute ethanol (200µl) was added to each sample and then added to DNA extraction columns. Following manufacturer's instructions, DNA extraction was carried out and extracted DNA was then suspended in Elution Buffer (Qiagen). Until analysis, the extracted DNA samples were stored at -20ºC. The quality of the extracted DNA was analysed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Loughborough).

2.8 Real-Time PCR

A pan-Chlamydiales qPCR targeting the 16S rRNA gene was performed to quantify the replication of the organism in culture. The qPCR assays were performed as previously described by Lienard *et al*. (Lienard *et al.*, 2011). using the forward primer panCH16F2 (5´-CCGCCAACACTGGGACT-3´), the reverse primer panCh16R2 (*5′-GGAGTTAGCCGGTGCTTCTTTAC-3′*) and the probe panCh16S (5′-FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1-3′). Assays were performed in a total

volume of 20µl, using the PrecisionPlus 2X qPCR MasterMix Low ROX and inert blue dye (Primerdesign, Southampton, UK), 0.1µM primer (Exiqon, Vedbaek, Denmark), a 0.1µM probe (Integrated DNA Technologies, Iowa, USA), molecular biology grade water and 2µl DNA. The PCR products were detected with a StepOnePlus Real-Time PCR System (ThermoFisher Scientific, UK). Molecular grade water was used as a negative PCR control. Quantification was achieved using a standard curve derived using a recombinant plasmid control (purified from a *Parachlamydia acanthamoebae* bacterial culture) as previously described by Lienard *et al.* 2011.

2.9 Immunostaining of bacterial inclusions

Host cell monolayers grown in chamber slides were fixed with ice-cold acetone for 5 minutes. Acetone was removed by aspiration and the slides were left to air dry prior freezing at -20ºC for a minimum of 20 minutes. Slides were removed from −20°C storage and rehydrated in 1X PBS until room temperature (RT). The slide chambers were then removed for immunostaining. Slides were blocked in a 2% BSA in PBS solution for a minimum of 30 minutes at RT. The slides were washed 3X in PBS prior to addition of primary antibodies, rabbit anti-*W. chondrophila* serum and mouse anti-*C. trachomatis*-LPS overnight at 4^{o}C at a 1:2000 dilution (in 1X PBS). After a further washing step, the slides were incubated with the secondary antibody, a goat anti-rabbit TRITC and anti-mouse ALEXA 594 respectively, in a 1:1000 dilution (in 1X PBS) for 1 hour at RT. After another washing step, host cell actin filaments stain was performed using ALEXA 488 Green Phalloidin (Life technologies, Paisley, UK). Slides were washed and mounted using Vectashield anti-fade mounting medium containing DAPI (Vector Laboratories, Peterborough, UK). Slides were examined using a digital imaging system with an Axioscope fluorescent microscope (Carl Zeiss Ltd, UK) equipped with GFP, RFP and DAPI fluorescent filter sets for inclusion counting. For image capture slides were further examined using a LSM 800 confocal microscope (Carl Zeiss Ltd, UK).

2.10 Lactate Dehydrogenase assay

Recovered supernatants from experiments described in Results section 2.6 were analysed by transferring 50µl of the supernatant to a 96-well flat bottom plate in duplicate. Lactate

Dehydrogenase release from host cells was then measured by a CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega, Madison, USA). A positive control for LDH release was obtained by exposing uninfected host cell monolayers to 0.01% Triton-X in PBS. 50µl of reconstituted substrate mix, provided by kit, was added to each well and plate was incubated for 30 minutes at room temperature protected from light. The Stop solution (50µl) provided in the kit was added to each well and absorbance was recorded at 492nm on Dynex® microplate reader (Dynex Technologies, Worthing, UK). Absorbances were converted into % of LDH release, considering that the positive control (host cell monolayers treated with Triton-X) released 100% of available LDH.

2.11 IL-8 Enzyme Linked ImmunoSorbent Assay (ELISA)

Experiments described in Results sections 2.6 and 2.7, direct cathelicidin treatment of *C. trachomatis* and *W. chondrophila* and host cell exposure to cathelicidins prior infection, were performed using MOI 1 and MOI 0.1 for both *W. chondrophila* and *C. trachomatis* and supernatants were recovered after 24 hours post infection with *W. chondrophila* and *C. trachomatis* and after 48 hours post infection with *C. trachomatis*. Supernatants were analysed for the presence of IL-8 by Enzyme Linked ImmunoSorbent Assay (ELISA). The IL-8 ELISAs were performed using a DuoSet human CXCL8/IL-8 ELISA kit (R&D Systems, Abingdon, UK). All reagents were reconstituted and prepared following manufacturer's instructions and given concentrations. 96-well flat bottom plates were coated overnight with the capture antibody provided by the kit. Plates were washed with 1X PBS and blocked (1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered) for a minimum of 1 hour at room temperature (RT). After a washing step, 100µL of each sample was added. A seven-point standard curve was created diluting the reconstituted standard reagent 1:2 in Reagent Diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline, 20mM Trizma base, 150mM NaCl, pH 7.2-7.4, 0.2µm filtered) starting at a 3000pg/ml concentration. 100µL of Reagent Diluent was used as blank. All samples, standards and controls were added in duplicate. Supernatant samples were incubated at RT for 2 h prior washing step with 1X PBS. The working solution of detection antibody in Reagent Diluent was then added to each well and incubated at RT for 2 hours. The plate was washed and the working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes at RT. After a further washing step, substrate solution

(TMB, ThermoFisher Scientific, UK) was then added to form an enzyme-antibody-target complex to produce a measurable signal. After 20 minutes the substrate formation was stopped by adding 50µL of Stop solution (2N H_2SO_4). The optical density of each well was determined immediately, using a microplate reader (Dynex®, Dynex Technologies, Worthing, UK) set to 450nm with wavelength correction set to 540nm (this subtraction will correct for optical imperfections in the plate).

3 Results

3.1 The direct antimicrobial effects of cathelicidins on C. trachomatis and W. chondrophila

3.1.1 Exposure to LL-37, PG-1 or SMAP-29 reduces Chlamydia trachomatis infectivity

To assess whether cathelicidins alter the infectivity of *C. trachomatis*, bacteria were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to host cell infection. sLL-37, a control peptide with identical amino acid composition but scrambled sequence, was used to determine the importance of sequence specificity in the peptide activity. At a time point of 24 hours post infection, host cell monolayers were fixed. Bacterial inclusions were then immunostained and visualised inside the host cells (Figure 9). The number of intracellular inclusions in each treatment was then quantified and represented. A gradual decrease in the number of visible inclusions was observed, indicating a reduction in *C. trachomatis* infectivity in the presence of LL-37, PG-1 or SMAP-29 treatment (Figure 10A, 10B and 10C). All peptide treatments resulted in a statistically significant reduction in the number of inclusions, at varying concentrations of each peptide used. PG-1 appeared to display the most effective antimicrobial activity at concentrations as low as 10μg/ml, whereas LL-37 and SMAP-29 did demonstrate antimicrobial activity, but at higher concentrations.

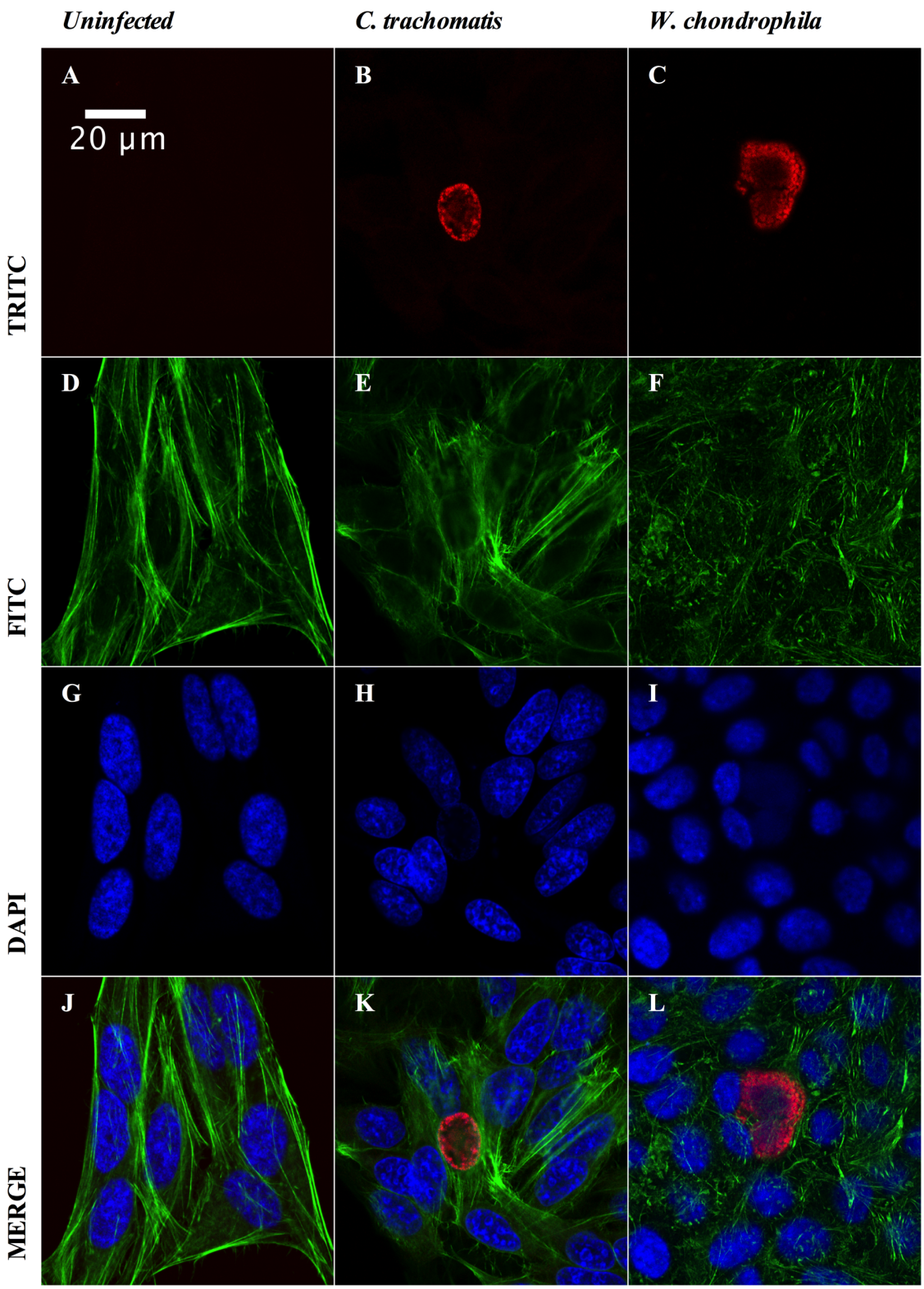


Figure 9. Confocal imaging of the intracellular localisation of bacterial inclusions. The human epithelial cell line, HEp2, was infected with *C. trachomatis* or *W. chondrophila* and fixed with acetone after 24h. Specific immunostaining of the bacterial inclusions was performed (A, B and C). Host cell actin filaments were stained by

phalloidin (D, E and F) and a DAPI stain of host cell nuclei was performed while mounting the slides (G, H and I). Images were generated on a LSM800 Zeiss microscope.

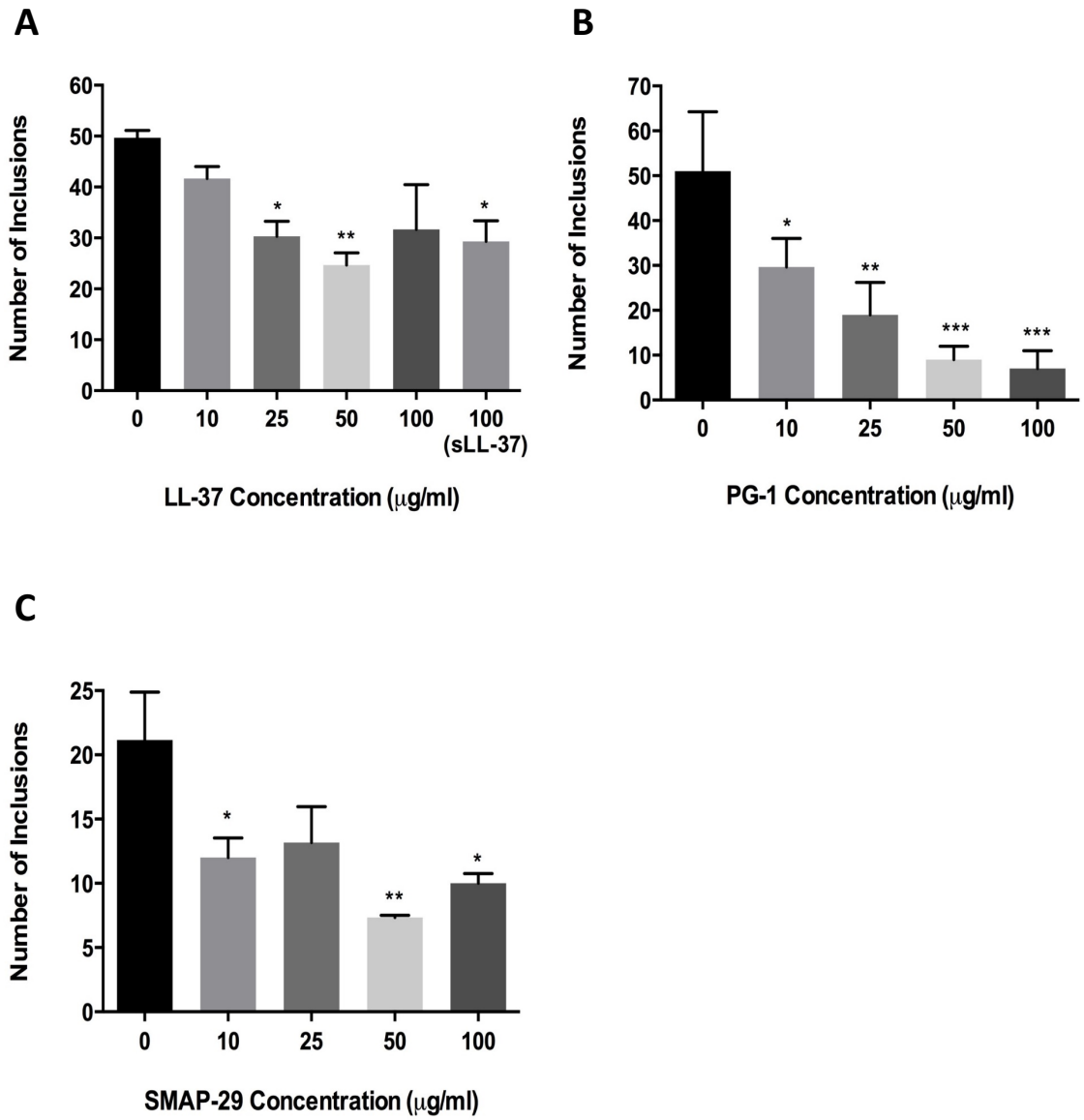


Figure 10. Average number of quantifiable inclusions after exposure of *Chlamydia trachomatis* to cathelicidins prior to host cell infection. *C. trachomatis* was incubated for 1 hour with a range of peptide concentrations prior to infection of HEp2 cell monolayers for an additional 1 hour. After 24 hours, immunostaining and counting of the inclusions was performed using an inverted fluorescence microscope. Data represents the average number of inclusions counted per treatment ± SEM (n=3). Statistical analysis was performed via a one-way ANOVA

with Dunnett's *post-hoc* test comparing *C. trachomatis* treated samples to the untreated control (0μg/ml). *p≤0.05, **p≤0.01, *** p≤0.001.

3.1.2 Exposure to LL-37, PG-1 and SMAP-29 reduces Chlamydia trachomatis replication

To assess whether cathelicidins alter replication of *C. trachomatis*, the bacteria were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to host cell infection. Following infection, bacterial ribosomal 16S gene copies were quantified by qPCR, revealing a decrease in bacterial replication in treatments where bacteria were exposed to high concentrations of peptide. (Figures 11A, 11B and 11C). Bacterial infection of the cells was highly variable, with measurable average gene copy number varying between 19000 copies (0μg/ml SMAP-29) to 120 copies (100μg/ml PG-1), likely due to a higher sensitivity to PG-1 exposure, also seen in the dramatic decrease of *C. trachomatis* infectivity after PG-1 exposure (Figure 9B). While LL-37 exposure only resulted in a moderate decrease in bacterial gene copy number measured, the ovine cathelicidin SMAP-29 induced statistically significant decreases in gene copy number even at the low concentration of 25μg/ml. The porcine cathelicidin PG-1 was also moderately effective at reducing 16S gene copy number, although this was not as successful as SMAP-29, and was not statistically significant.

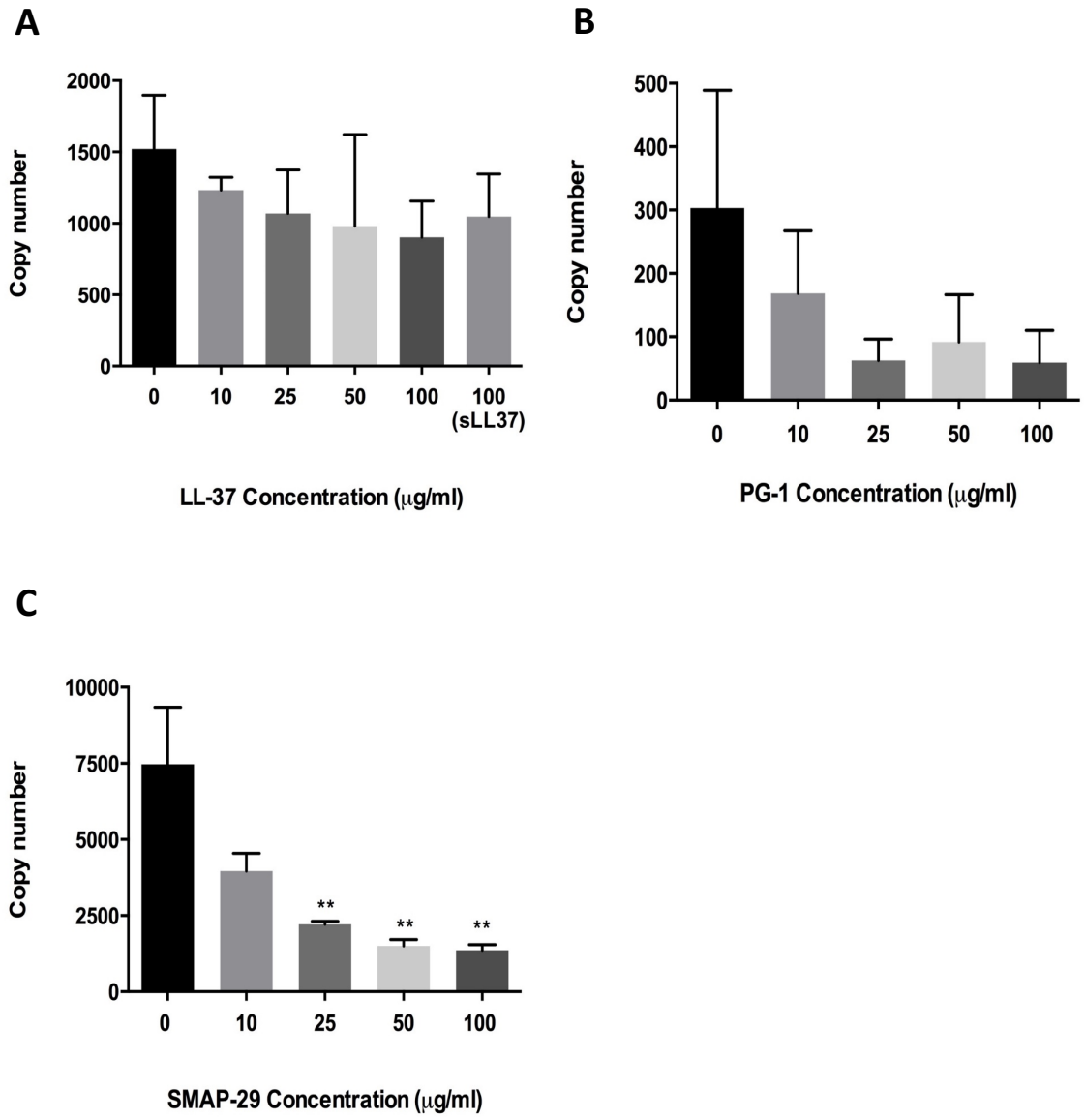


Figure 11. Average Chlamydiales 16S gene copy number after exposure of *Chlamydia trachomatis* to cathelicidins prior to host cell infection. *C. trachomatis* was exposed for 1 hour to varying peptide concentrations prior to infection of HEp2 cell monolayers for an additional 1 hour. After 24 hours, DNA was extracted from cell monolayers and quantitative PCR was performed to assess 16S gene copy number. Data shown reflects a correction in the average bacterial copy number as each organism presents two copies of 16S gene. Consequently, the obtained copy numbers were divided by two. Figures represent the mean ± SEM (n=3). Statistical significance was assessed by one-way ANOVA with Dunnett's *post-hoc* test comparing peptide treated samples to the untreated control (0μg/ml). A p value of ≤ 0.05 was considered significant. (**p≤0.01).

3.1.3 Exposure to LL-37, PG-1 or SMAP-29 increases Waddlia chondrophila infectivity

To assess whether cathelicidins alter the infectivity of *W. chondrophila*, the bacteria were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to host cell infection. 24 hours post infection, host cell monolayers were fixed. The number of intracellular inclusions in each treatment was then quantified and represented. An increase in the number of inclusions was observed after exposure to cathelicidins, indicating a higher infectivity as a result of LL-37, PG-1 and SMAP-29 treatment (Figure 12A, 12B and 12C). This statistically significant increase occurred at all concentrations of LL-37 and PG-1 although SMAP-29 did not induce significant increases at concentrations below 50μg/ml.

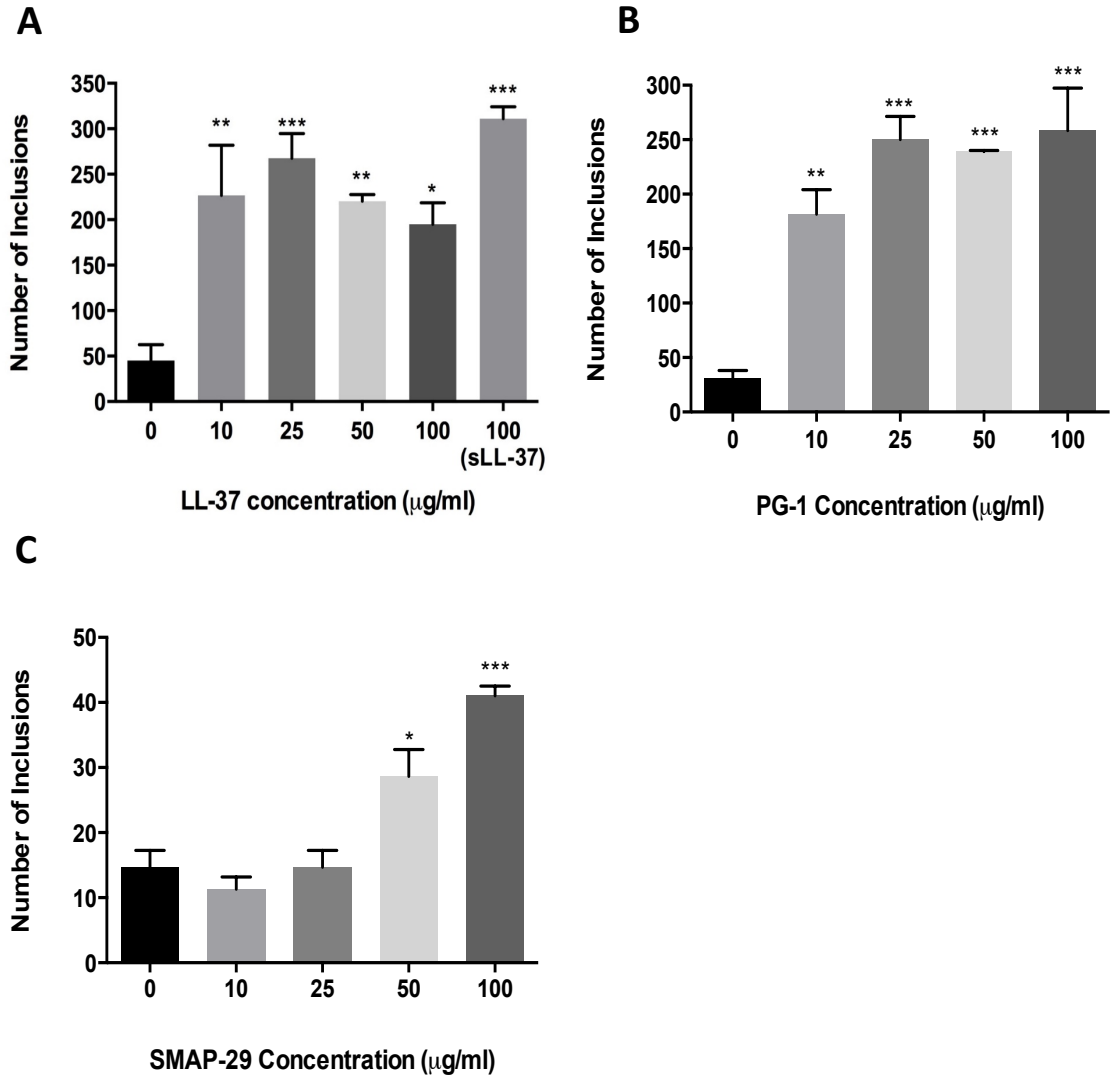


Figure 12. Average number of inclusions after cathelicidin incubation with *Waddlia chondrophila* prior to host cell infection. *W. chondrophila* was pre-incubated for 1 hour with different peptide concentrations prior addition to HEp2 cell monolayers for another 1 hour incubation. At 24 hours post infection, immunostaining and counting of the inclusions was performed using an inverted fluorescence microscope. Data represents the average number of inclusions per treatment ± SEM (n=3). Significance was determined by one-way ANOVA with Dunnett's *post-hoc* test comparing peptide treated samples to the untreated control (0μg/ml). *p≤0.05, **p≤0.01, *** p≤0.001.

3.1.4 Exposure to LL-37, PG-1 or SMAP-29 increases W. chondrophila replication

Following the same approach as previously adopted with *C. trachomatis*, the effect of cathelicidin exposure on the replication of *W. chondrophila* was assessed. *W.*

chondrophila was directly exposed to varying concentrations of either LL-37, PG-1 or SMAP-29 peptides prior infection of host cells. Following infection, the bacterial ribosomal 16S gene copies were quantified by qPCR, and revealed an increase in bacterial replication in response to peptide treatments. (Figure 13A, 13B and 13C). A high variability in total bacterial infection numbers was observed in the peptide treated samples. Exposure to LL-37 and scrambled LL-37 resulted in statistically significant increases in gene copy number at concentrations of 25μg/ml or higher (Figure 13A). Exposure to PG-1 also increased measurable 16S gene copy number in all treatments, although due to the variability of the measured gene copy numbers, this was not statistically significant. SMAP-29 exposure also appears to induce an increase in gene copy number.

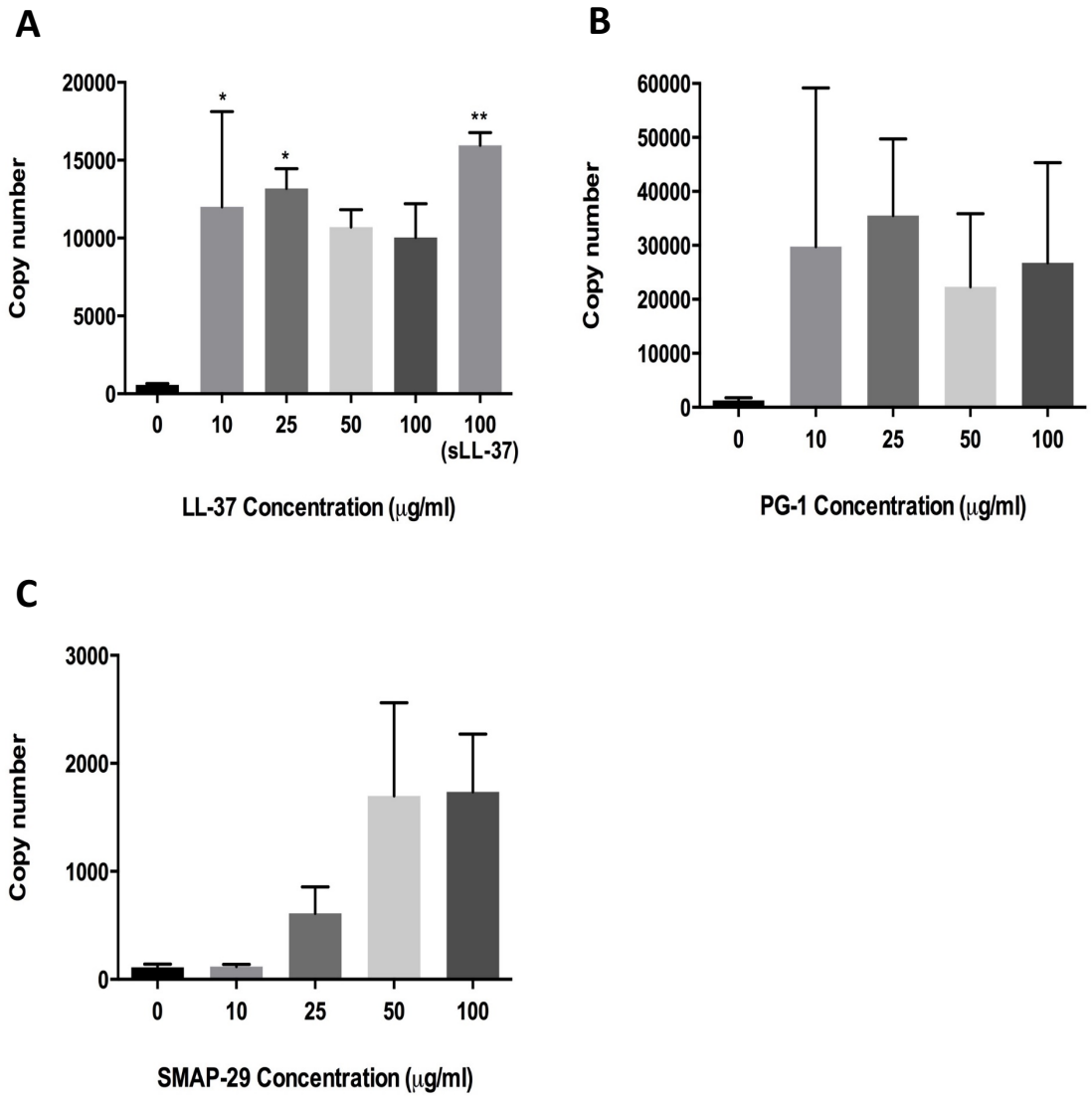


Figure 13. Average Chlamydiales 16S gene copy number after exposure of *Waddlia chondrophila* to cathelicidins prior to host cell infection. *W. chondrophila* was exposed for 1 hour to varying peptide concentrations prior to infection of HEp2 cell monolayers for an additional 1 hour. After 24 hours, DNA was extracted from cell monolayers and quantitative PCR was performed to assess 16S copy number. Data shown reflects a correction in the average bacterial copy number as each organism presents two copies of 16S gene. Consequently, the obtained copy numbers were divided by two. Figures represent the mean ± SEM (n=3). Statistical analysis was performed by one-way ANOVA and Dunnett´s post-hoc test comparing *Waddlia chondrophila* treated samples to the untreated control (0μg/ml). *p≤0.05, **p≤0.01.

3.2 The effect of host cell exposure to cathelicidins on C. trachomatis and W. chondrophila infection

3.2.1 Host cell exposure to LL-37, but not PG-1 or SMAP-29, alters subsequent *C. trachomatis* infectivity

To assess whether cathelicidin pre-treatment of host cells alter infectivity of *C. trachomatis*, HEp2 cells were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to *C. trachomatis* infection. Following infection and incubation, host cell monolayers were fixed and bacterial inclusions were then specifically stained and visualised inside the host cells. The number of intracellular inclusions in each treatment was then quantified and represented (Figure 14A, 14B and 14C). The obtained number of inclusions per treatment for each cathelicidin, did not reveal a clear effect on *C. trachomatis* infectivity as the number of inclusions was similar among the different concentrations in each treatment.

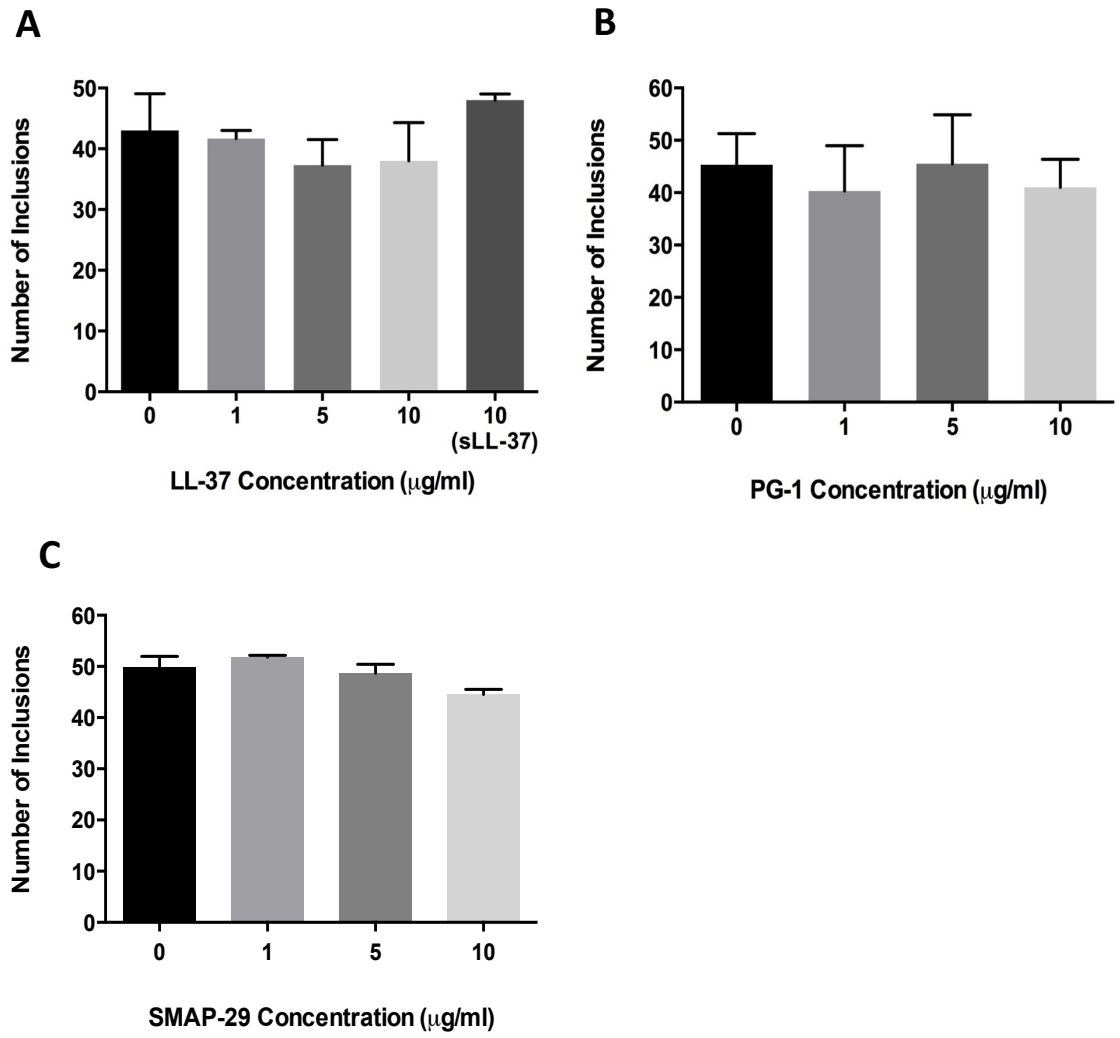


Figure 14. Average number of inclusions after host cell exposure to cathelicidins prior to *C. trachomatis* infection. Host cells were incubated for 1 hour with a range of peptide concentrations prior to infection with *C. trachomatis* for an additional 1 hour. After 24 hours, specific immunostaining and counting of the inclusions was then performed. Data represents the average number of inclusions per treatment ± SEM (n=3). Significance was determined by one-way ANOVA with Dunnett's *post-hoc* test comparing peptide treated samples to the untreated control (0μg/ml). A p value ≤0.05 was considered significant.

3.2.2 Host cell exposure to LL-37, PG-1 or SMAP-29 does not alter C. trachomatis replication

To study whether cathelicidin pre-treatment of host cells alters replication of *C. trachomatis*, HEp2 cells were directly exposed to varying concentrations of LL-37, PG-

1 and SMAP-29 for 1 hour prior to *C. trachomatis* infection. Following infection and incubation, the bacterial ribosomal 16S gene copies were quantified by qPCR, and revealed a decrease in bacterial replication in treatments where bacteria were exposed to high concentrations of peptide (Figures 15A, 15B and 15C). While PG-1 and SMAP-29 exposure only resulted in a moderate decrease in the bacterial gene copy number, the human cathelicidin LL-37 induced a significant decrease at the highest concentration (10μg/ml).

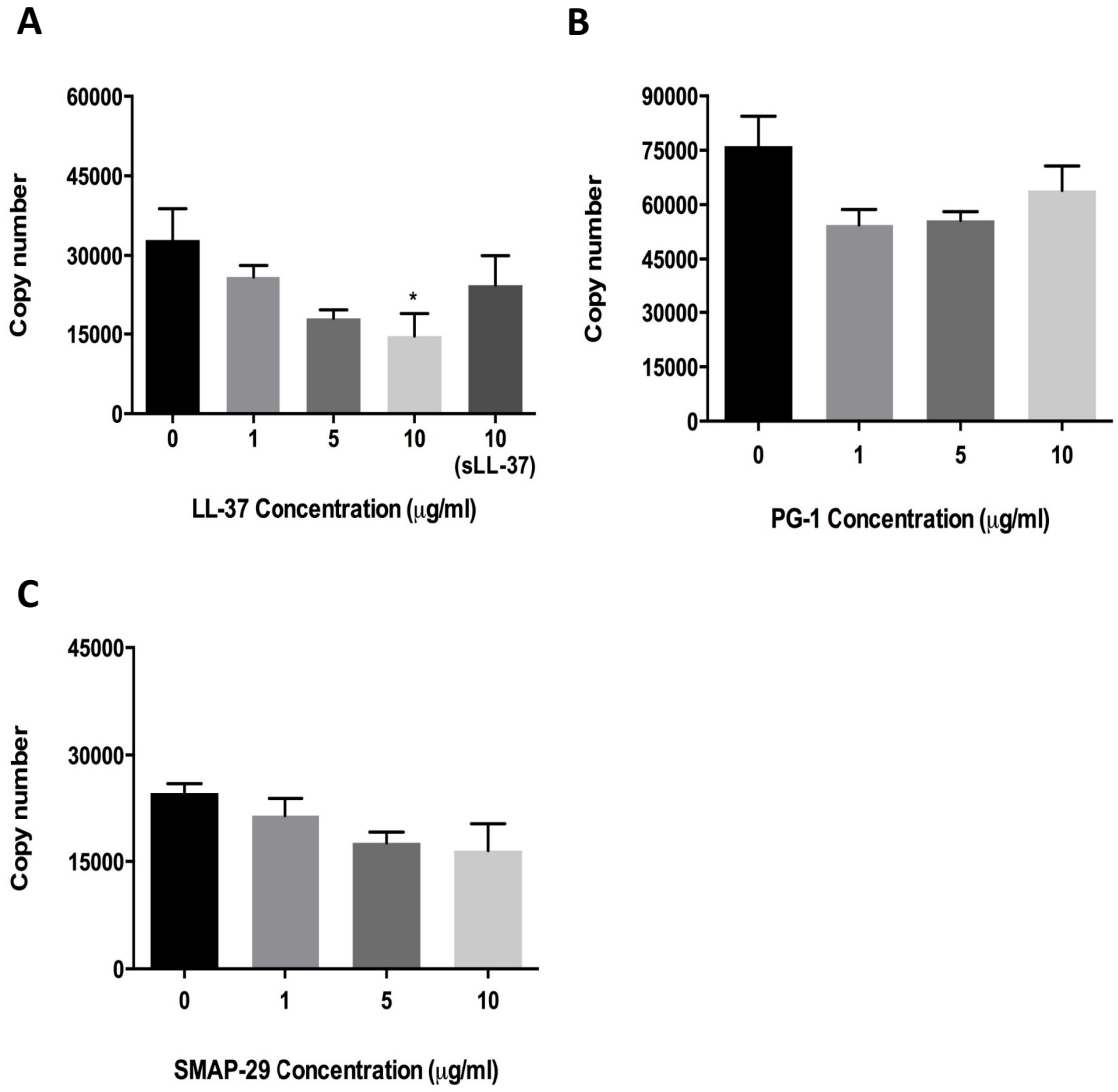


Figure 15. Average Chlamydiales 16S gene copy number after host cell exposure to cathelicidins prior to *C. trachomatis* infection. Host cells were incubated for 1 hour with a range of peptide concentrations prior to infection with *C. trachomatis* for an additional 1 hour. After 24 hours, DNA was extracted from cell monolayers and quantitative PCR was performed to assess 16S copy number. Data shown reflects a correction in the average bacterial copy number as each

organism presents two copies of 16S gene. Consequently, the obtained copy numbers were divided by two. Each copy number corresponds to one organism and figures represent mean ± SEM (n=3). Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test comparing peptide treated samples to the untreated control (0µg/ml). *p≤0.05.

3.2.3 Host cell exposure to LL-37, PG-1 or SMAP-29 does not alter W. chondrophila infectivity

To assess whether cathelicidin pre-treatment of host cells alters infectivity of *W. chondrophila,* HEp2 cells were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to *W. chondrophila* infection. Following infection and incubation, host cell monolayers were fixed and bacterial inclusions were then specifically stained and visualised inside the host cells. The average number of inclusions revealed minor decreases in *W. chondrophila* infectivity, following LL-37 and PG-1 exposure, but this was not statistically significant (Figures 16A and 16B). SMAP-29 exposure did not alter infectivity (Figure 16C).

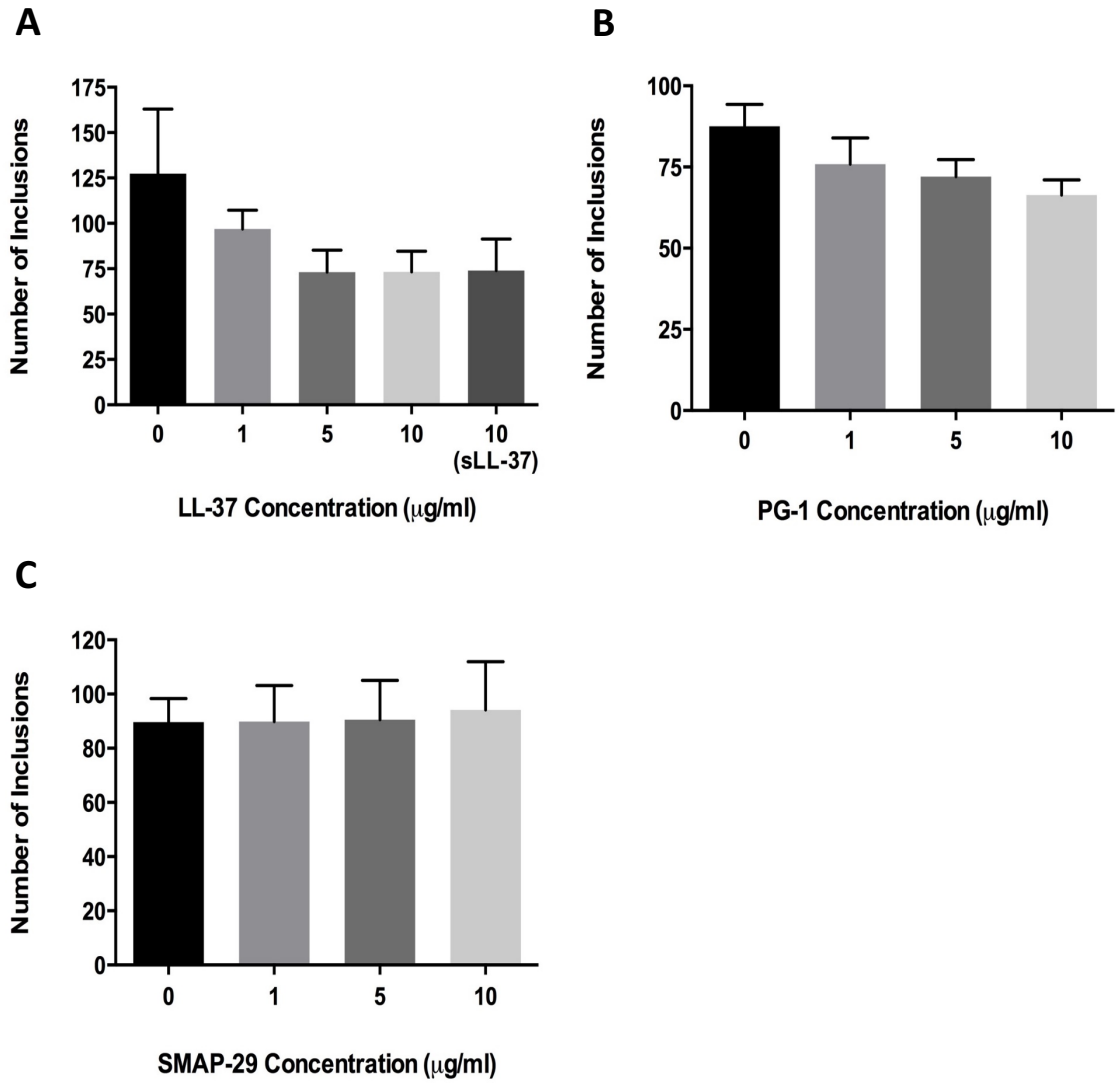


Figure 16. Average number of inclusions after host cell exposure to cathelicidins prior to *Waddlia chondrophila* infection. HEp2 cell monolayers were exposed for 1 hour to various peptide concentrations prior to infection with *Waddlia chondrophila* for an additional 1 hour. After 24 hours, specific immunostaining and counting of the inclusions was performed using an inverted fluorescence microscope. Data represents the average number of inclusions counted per treatment ± SEM (n=3). Significance was determined by one-way ANOVA with Dunnett's *post-hoc* test comparing *W. chondrophila* treated samples to the untreated control (0μg/ml). A *p* value of ≤0.05 was considered significant.

3.2.4 Host cell exposure to LL-37, PG-1 or SMAP-29 does not alter W. chondrophila replication

To study whether cathelicidin pre-treatment of host cells alters replication of *W. chondrophila*, HEp2 cells were directly exposed to varying concentrations of LL-37, PG-1 and SMAP-29 for 1 hour prior to *W. chondrophila* infection. Following infection and incubation, the bacterial ribosomal 16S gene copies were quantified by qPCR. Host cell exposure to cathelicidins did not reveal a significant increase or decrease of *W. chondrophila* replication at the used concentrations of LL-37, PG-1 and SMAP-29 (Figure 17A, 17B and 17C). A similar trend is seen in Figure 17A and 17B, where exposure to 5 μg/ml of PG-1 and LL-37 decreased moderately bacterial copy number. On the contrary, exposure to lower concentrations of SMAP-29 resulted in a similar copy number, being only the highest concentration the one that promoted a higher replication.

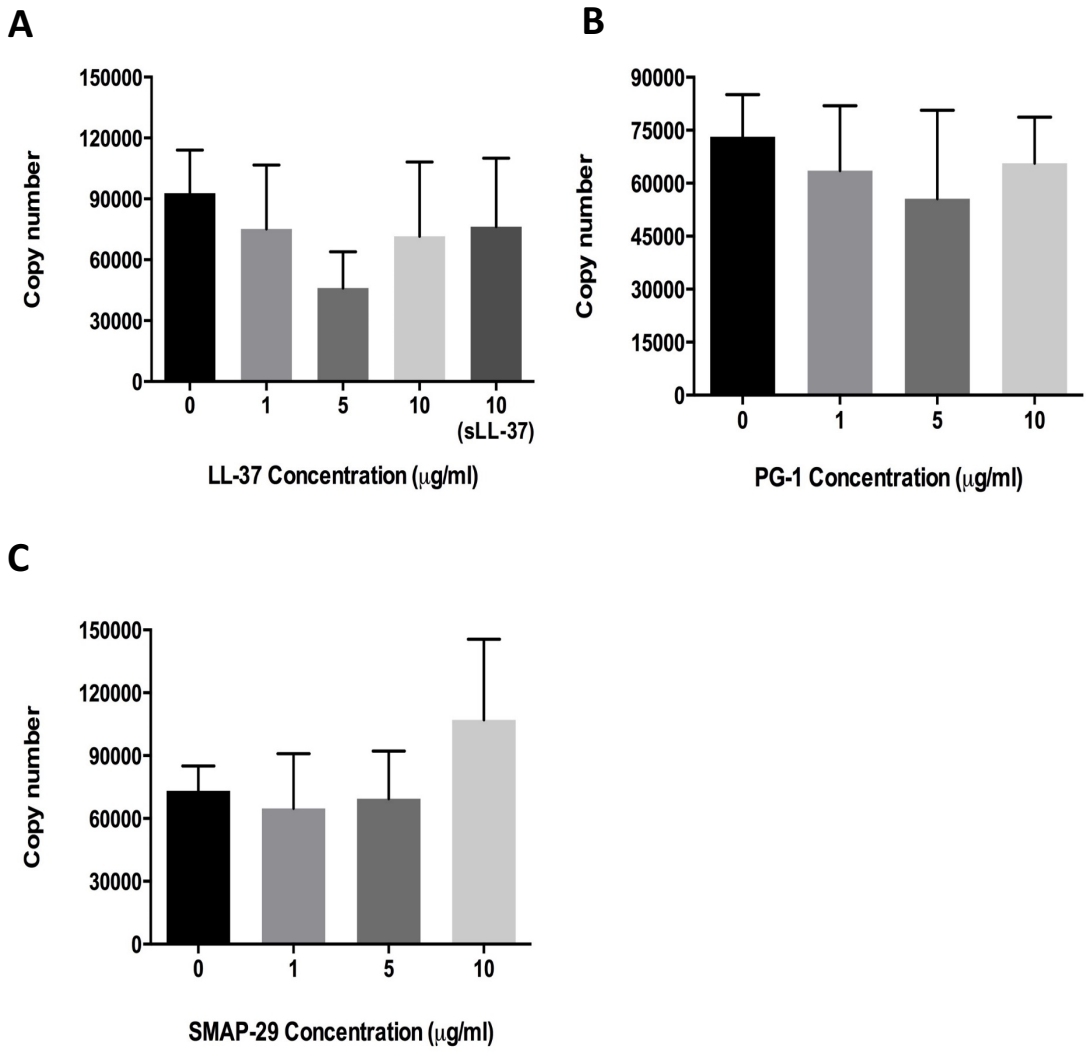


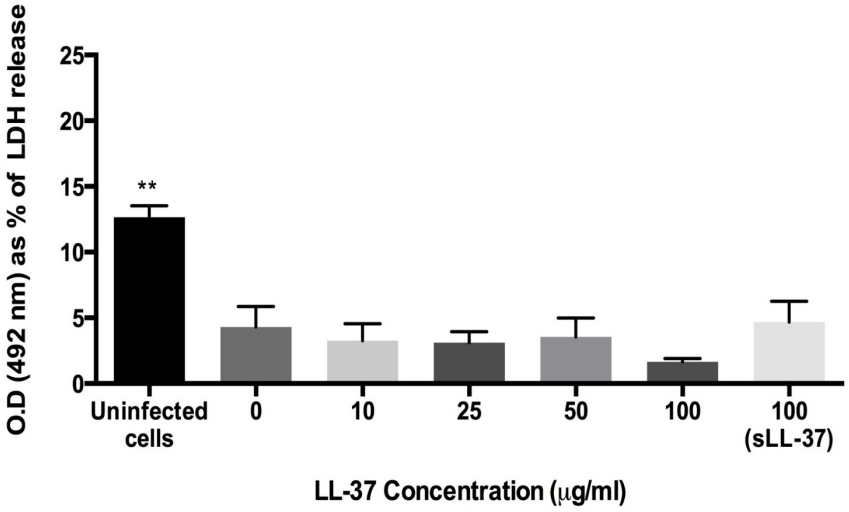
Figure 17. Average Chlamydiales 16S gene copy number after host cell exposure to cathelicidins prior to *W. chondrophila* infection. Host cells were pre-incubated for 1 hour with a range of peptide concentrations prior to infection for an additional 1 hour. After 24 hours, DNA was extracted from cell monolayers and qPCR was performed to assess 16S copy number. Data shown reflects a correction in the average bacterial copy number as each organism presents two copies of 16S gene. Consequently, the obtained copy numbers were divided by two. Figures represent mean ± SEM (n=3). Statistical significance was assessed by one-way ANOVA with Dunnett's *post-hoc* test comparing peptide treated samples to the untreated control (0μg/ml). A *p* value of ≤ 0.05 was considered significant.

3.3 The effect of cathelicidin exposure on host cell death

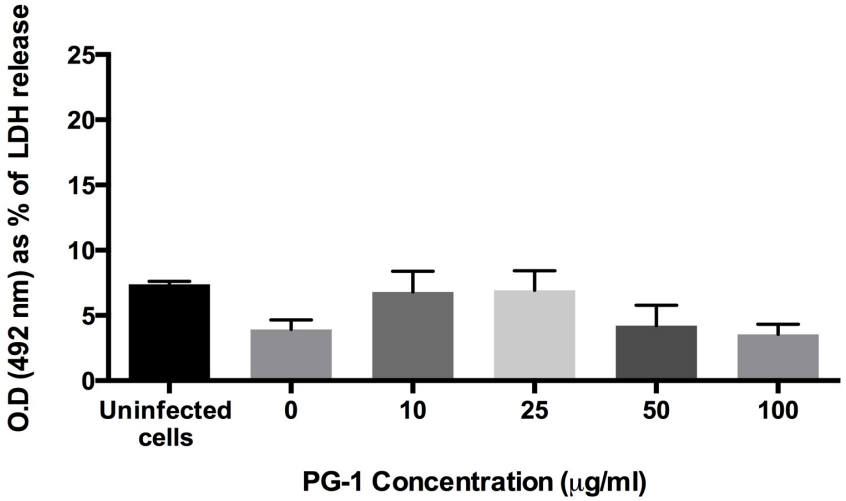
3.3.1 Exposure to LL-37, PG-1 and SMAP-29 does not induce necrosis in C. trachomatis infected host cells

To determine if cathelicidin exposure induced host cell death in *C. trachomatis* infected HEp2 cells, supernatants were analysed for the presence of the intracellular enzyme lactate dehydrogenase, indicative of necrotic cell death. The supernatants analysed were obtained following direct cathelicidin treatment of *C. trachomatis* prior to host cell infection (Results section 1) and represented 24 hours post-infection. Supernatants were recovered 24 hours post infection with bacteria that had been directly exposed to peptides prior incubation with host cells. Only when *C. trachomatis* was exposed to 0μg/ml SMAP-29, the LDH release was significantly higher than when host cells were uninfected. Bacterial exposure to LL-37, PG-1 and SMAP-29 did not resulted in an increase of host cell death, even when bacteria was exposed to higher concentrations of peptides (Figure 18A, 18B and 18C).

A


O.D (492 nm) as % of LDH release
25
20
15
10
5
0
**
Uninfected cells
0
10
25
50
100
100 (sLL-37)
LL-37 Concentration (μg/ml)

B


O.D (492 nm) as % of LDH release
25
20
15
10
5
0
Uninfected cells
0
10
25
50
100
PG-1 Concentration (μg/ml)

C

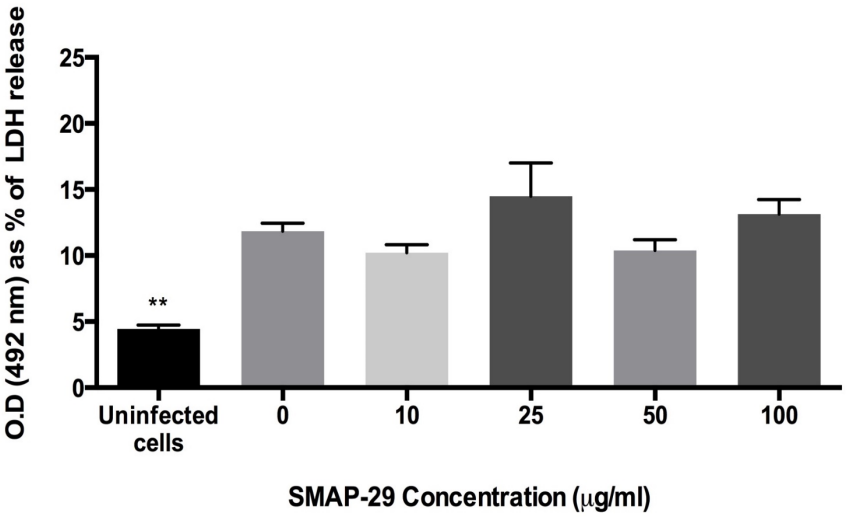
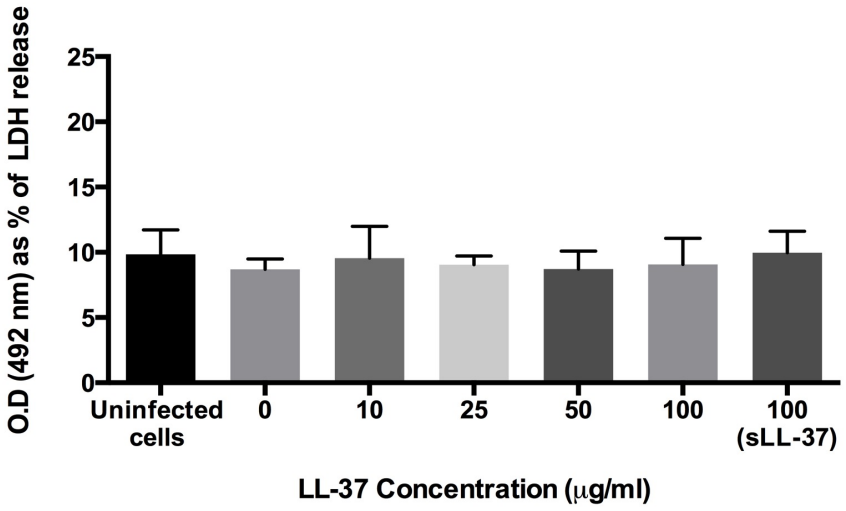

O.D (492 nm) as % of LDH release
25
20
15
10
5
0
**
Uninfected cells
0
10
25
50
100
SMAP-29 Concentration (μg/ml)

Figure 18. Average LDH release from host cells following infection with *C. trachomatis* that had previously been exposed to cathelicidins. *C. trachomatis* was exposed for 1 hour to different cathelicidin concentrations prior host cell infection for a further 1 hour. Following a wash step and 24 hours incubation, supernatants were collected and LDH release was determined using a CytoTox 96® Non-Radioactive Cytotoxicity kit. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett multiple comparison test to compare each treatment to the infected untreated control (0μg/ml). A *p* value of ≤0.05 was considered significant. **p≤0.01.

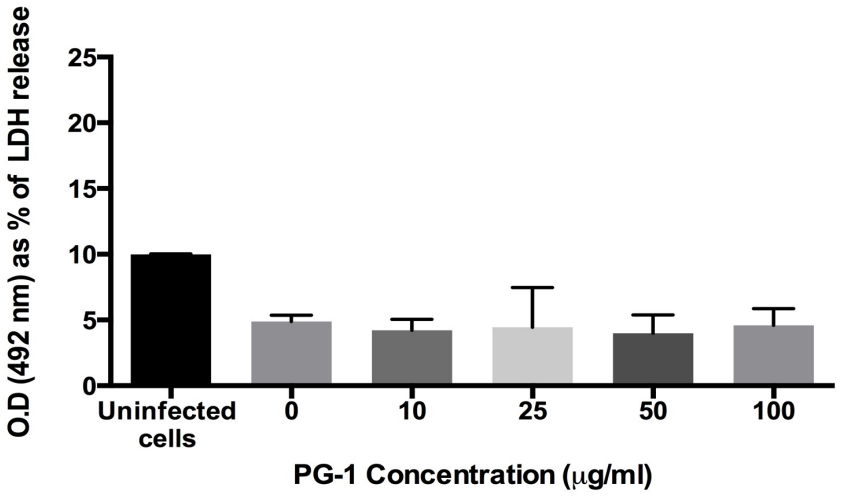
3.3.2 Exposure to LL-37, PG-1 and SMAP-29 does not induce necrosis in W. chondrophila infected host cells

To determine if cathelicidins induced host cell death in *W. chondrophila* infected HEp2 cells, supernatants recovered after 24 hours incubation with *W. chondrophila* were analysed by CytoTox 96® Non-Radioactive Cytotoxicity kit to study the host cell LDH release (Figure 19A, 19B and 19C). Detectable LDH release was not higher in any of the analysed samples compared to the uninfected negative control, even at higher concentrations of cathelicidin.

A


O.D (492 nm) as % of LDH release
25
20
15
10
5
0
Uninfected cells
0
10
25
50
100
100 (sLL-37)
LL-37 Concentration (μg/ml)

B


O.D (492 nm) as % of LDH release
25
20
15
10
5
0
Uninfected cells
0
10
25
50
100
PG-1 Concentration (μg/ml)

C

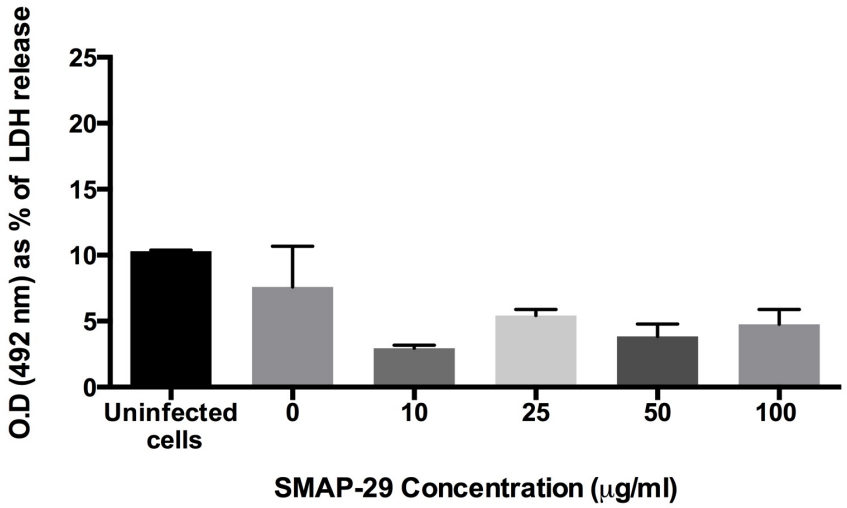

O.D (492 nm) as % of LDH release
25
20
15
10
5
0
Uninfected cells
0
10
25
50
100
SMAP-29 Concentration (μg/ml)

Figure 19. Average LDH release from host cells following infection with *W. chondrophila* that had previously been exposed to cathelicidins. *W. chondrophila* was exposed for 1 hour to different cathelicidin concentrations prior to host cell infection for a further 1 hour incubation. Following a washing step and 24 hours incubation, supernatants were collected and LDH release was determined using a CytoTox 96® Non-Radioactive Cytotoxicity kit. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test to compare each treatment to the infected untreated control (0μg/ml). A p value of ≤0.05 was considered significant.

3.4 The effect of cathelicidins on the host inflammatory response during C. trachomatis and W. chondrophila infection

3.4.1 Modulation of IL-8 release by cathelicidins in uninfected host cells

3.4.1.1 Exposure to LL-37, PG-1 and SMAP-29 does not alter IL-8 release by HEp2 cells

Cathelicidins have been shown to elicit a host response to infection partly through their ability to modulate immune and inflammatory responses. HEp2 cells were exposed to peptides at varying concentrations for 1 hour, then washed and incubated for 24 h. Supernatants were analysed by Enzyme-Linked Immunosorbent Assay (ELISA). No significant increase or decrease in the IL-8 production was found after LL-37, PG-1 and SMAP-29 exposure (Figure 20A, 20B and 20C) when compared to untreated cells.

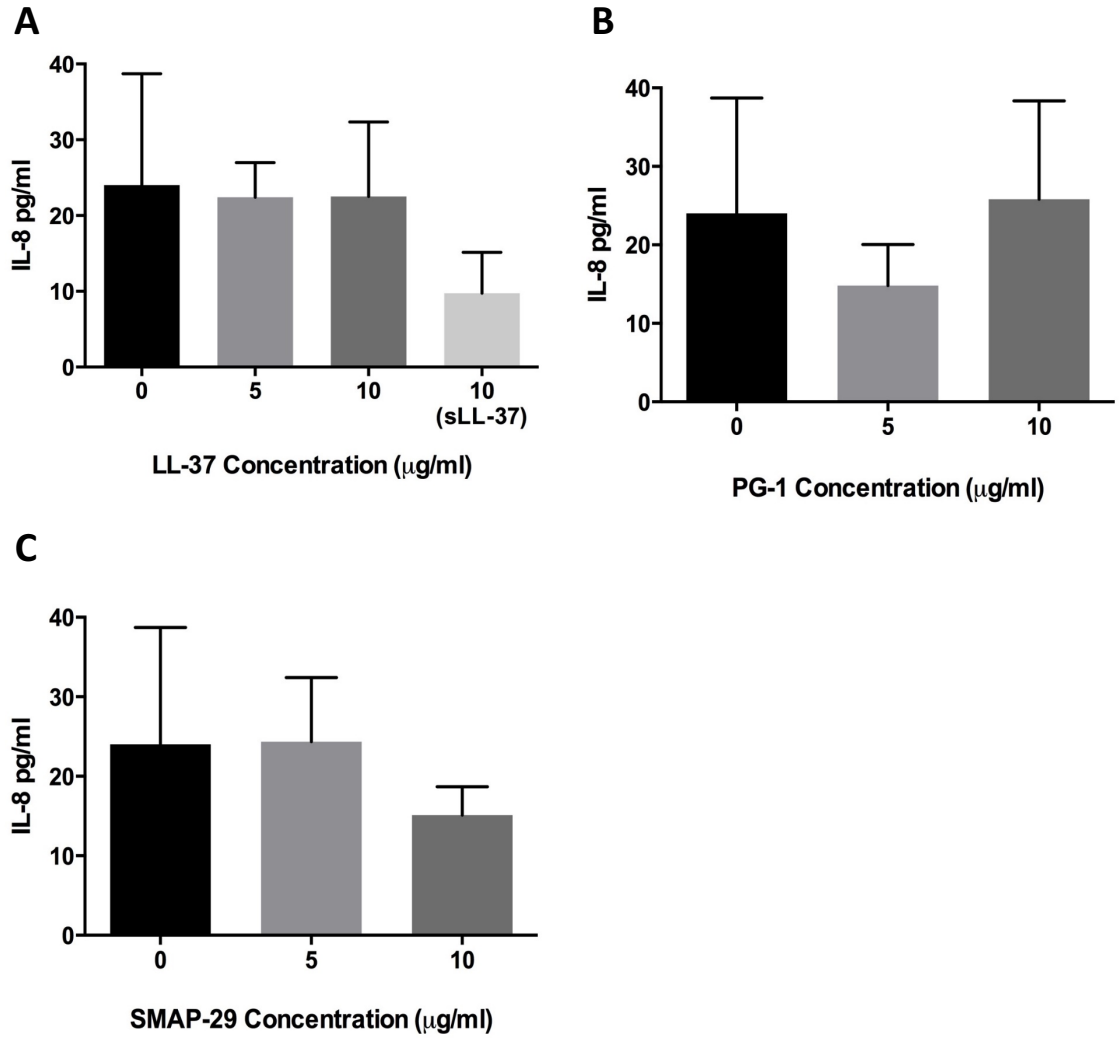


Figure 20. IL-8 release (pg/ml) by HEp2 cells following exposure to cathelicidins. HEp2 host cells were exposed to different cathelicidin concentrations for 1 h. Following a wash step, cells were incubated for 24h at 37ºC. Supernatants were collected and analysed, and results are shown as a mean ± SEM of three independent experiments. Significance was determined by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the rest. A p value of ≤0.05 was considered significant.

3.4.2 The effect of cathelicidins on IL-8 production in C. trachomatis infection

3.4.2.1 Cathelicidins dramatically enhance IL-8 release by host cells during C. trachomatis infection

C. trachomatis was exposed for 1h to various cathelicidin concentrations and incubated with the host cell for a further 1h. After a washing step and subsequent 24 h incubation, supernatants were recovered at 24 h and 48 h time points and analysed by ELISA to determine IL-8 release by host cells (Figures 21A-21F). At 24 h, there was an increase in IL-8 production by host cells in response to infection. When the bacteria were exposed to LL-37, PG-1 and SMAP-29, there was no significant alteration on IL-8 release compared to either the uninfected cells or the bacteria minus peptide treatments. However, at 48 h post infection, cathelicidin treatment of *C. trachomatis* promoted substantial IL-8 release by the host cells comparing to the non-peptide treated bacteria. LL-37 and SMAP-29 induced a statistically significant increase in IL-8 production. PG-1 also induced an increase in the host IL-8 production though it was not significant. Results from *C. trachomatis* MOI 0.1 were very low and are shown in the Results Appendix 5.1.1.

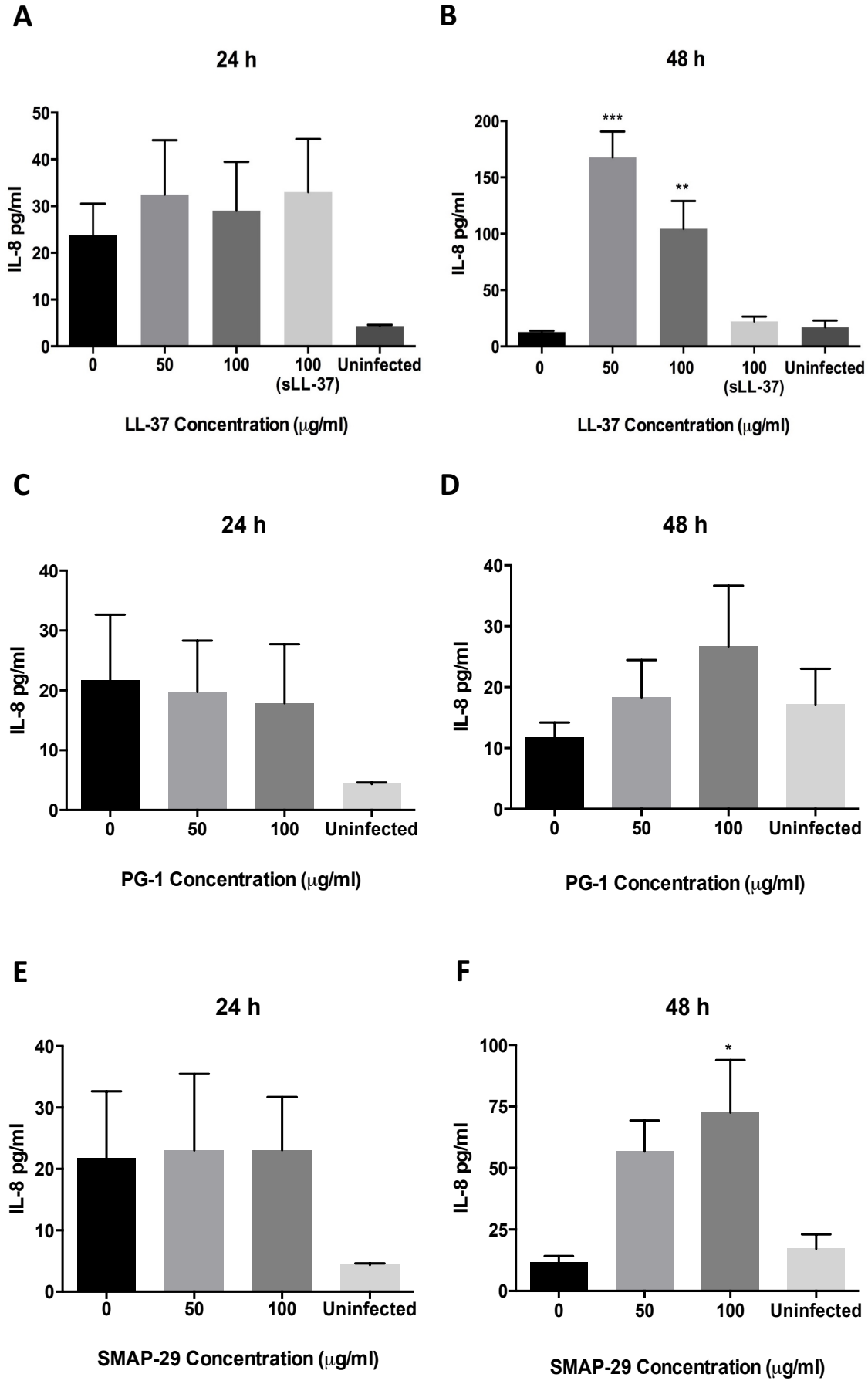

A
24 h
IL-8 pg/ml
50
40
30
20
10
0
0
50
100
100 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
B
48 h
IL-8 pg/ml
200
150
100
50
0
***
**
0
50
100
100 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
C
24 h
IL-8 pg/ml
40
30
20
10
0
0
50
100
Uninfected
PG-1 Concentration (μg/ml)
D
48 h
IL-8 pg/ml
40
30
20
10
0
0
50
100
Uninfected
PG-1 Concentration (μg/ml)
E
24 h
IL-8 pg/ml
40
30
20
10
0
0
50
100
Uninfected
SMAP-29 Concentration (μg/ml)
F
48 h
IL-8 pg/ml
100
75
50
25
0
*
0
50
100
Uninfected
SMAP-29 Concentration (μg/ml)

Figure 21. IL-8 release (pg/ml) after *C. trachomatis* (MOI 1) exposure to cathelicidins. *C. trachomatis* (MOI 1) was exposed for 1 hour to different cathelicidin concentrations prior to host cell infection for a further 1 hour. After 24 hours and 48 hours incubation, supernatants were collected and analysed by ELISA using uninfected cells as control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the untreated infected control (0μg/ml). *p≤0.05, ** p≤0.01, *** p≤0.001.

3.4.2.2 Host cell exposure to LL-37, PG-1 and SMAP-29 prior to C. trachomatis infection can alter IL-8 release

HEp2 cells were exposed to peptides for 1 hour and then infected with *C. trachomatis* (MOI 0.1 and 1) for a further 1 hour. Supernatants were recovered at 24 and 48 h and analysed by ELISA. Host cell treatment with LL-37 or SMAP-29 did not alter IL-8 production when compared to the infected control. However, when pre-exposing host cells to the porcine cathelicidin Protegrin-1, a reduction in IL-8 production by the host cells was observed at both peptide concentrations (Figures 22A, 22B and 22C). Results obtained from 24 h infection course of *C. trachomatis* MOI 0.1 can be found in the Results Appendix 5.1.2.

When host cells were infected with an MOI 1 of *C. trachomatis* IL-8 production was higher at 48 h post infection than 24 h post infection (Figure 23A-23F). Exposure to 5μg/ml of PG-1 and SMAP-29 showed a moderate increase in the IL-8 production comparing to the untreated sample at 24 h p.i. whereas this increase was not statistically significant with exposure to the pathogen only.

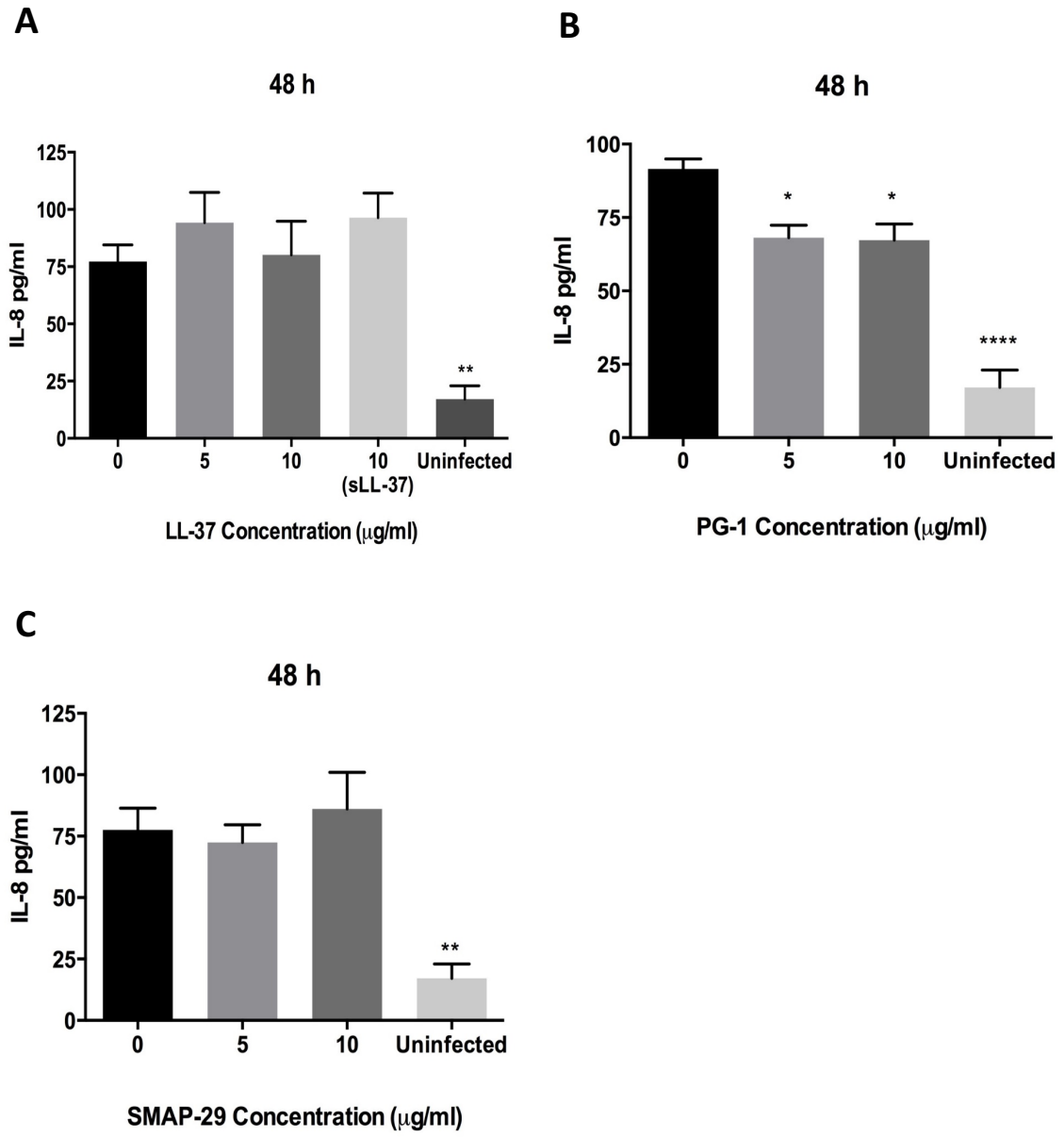


Figure 22. IL-8 release (pg/ml) after host cell exposure to cathelicidins prior to infection (MOI 0.1). HEp2 cells were pre-incubated for 1 hour with various cathelicidin concentrations and then infected for a further 1 hour with *C. trachomatis* (MOI 0.1). After 48 hours incubation, supernatants were collected and analysed using uninfected cells as control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the infected control (0μg/ml). *p≤0.05, ** p≤0.01, **** p≤0.0001.

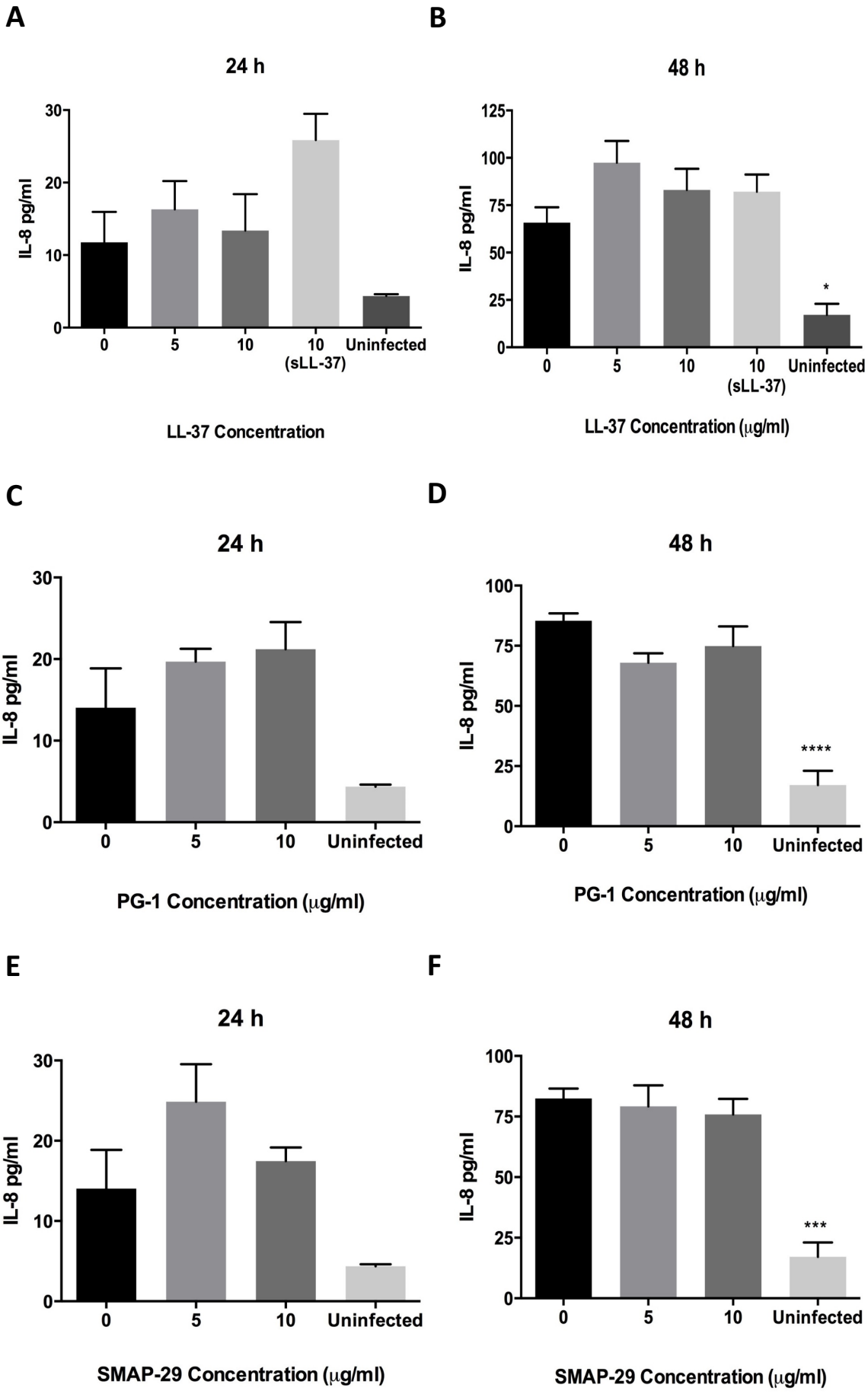

A
24 h
IL-8 pg/ml
30
20
10
0
0
5
10
10 (sLL-37)
Uninfected
LL-37 Concentration
B
48 h
IL-8 pg/ml
125
100
75
50
25
0
*
0
5
10
10 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
C
24 h
IL-8 pg/ml
30
20
10
0
0
5
10
Uninfected
PG-1 Concentration (μg/ml)
D
48 h
IL-8 pg/ml
100
75
50
25
0
****
0
5
10
Uninfected
PG-1 Concentration (μg/ml)
E
24 h
IL-8 pg/ml
30
20
10
0
0
5
10
Uninfected
SMAP-29 Concentration (μg/ml)
F
48 h
IL-8 pg/ml
100
75
50
25
0
***
0
5
10
Uninfected
SMAP-29 Concentration (μg/ml)

Figure 23. IL-8 release (pg/ml) after host cell exposure to cathelicidins prior to infection with *C. trachomatis* (MOI 1). HEp2 cells were incubated for 1 hour with various cathelicidin concentrations and then infected for a further 1 hour with *C. trachomatis.* After 24 hours and 48 hours incubation, supernatants were collected and analysed using infected cells without peptide treatment as a control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the infected control. * $p<0.05$, ***$p<0.001$, **** $p<0.0001$.

3.4.3 The effect of cathelicidins on IL-8 production in W. chondrophila infection

The effect of cathelicidins was also subjected to study in *Waddlia chondrophila* infection. As its infection cycle is shorter, the 24 h time point was enough to build an idea of the effects of the different treatments on the host cell inflammatory response through IL-8 production.

3.4.3.1 LL-37, PG-1 and SMAP-29 exposure alters W. chondrophila induced IL-8 production in epithelial host cells

W. chondrophila (MOI 0.1 and 1) was exposed for 1h to either LL-37, SMAP-29 or Protegrin-1 and the mix was added to HEp2 for a further 1 hour incubation. After a further 24 hours incubation, supernatants were recovered and analysed by ELISA (Figure 24A-24F). As expected, IL-8 production in response to *W. chondrophila* was observed to be higher at MOI 1 than at MOI 0.1. However, it was noted that IL-8 production was substantially higher when *W. chondrophila* had been pre-treated with cathelicidin, compared to infected control. This observation was only noted to be statistically significant when an MOI of 1 was utilized (Figures 24B, Figure 24D and Figure 24F).
At MOI 1 a significant difference between IL-8 production is seen when *W. chondrophila* has been exposed to cathelicidins than in the untreated bacteria. This difference is higher when *W. chondrophila* (MOI 1) was exposed to 50μg/ml and 100μg/ml of PG-1 and to 50μg/ml of LL-37.

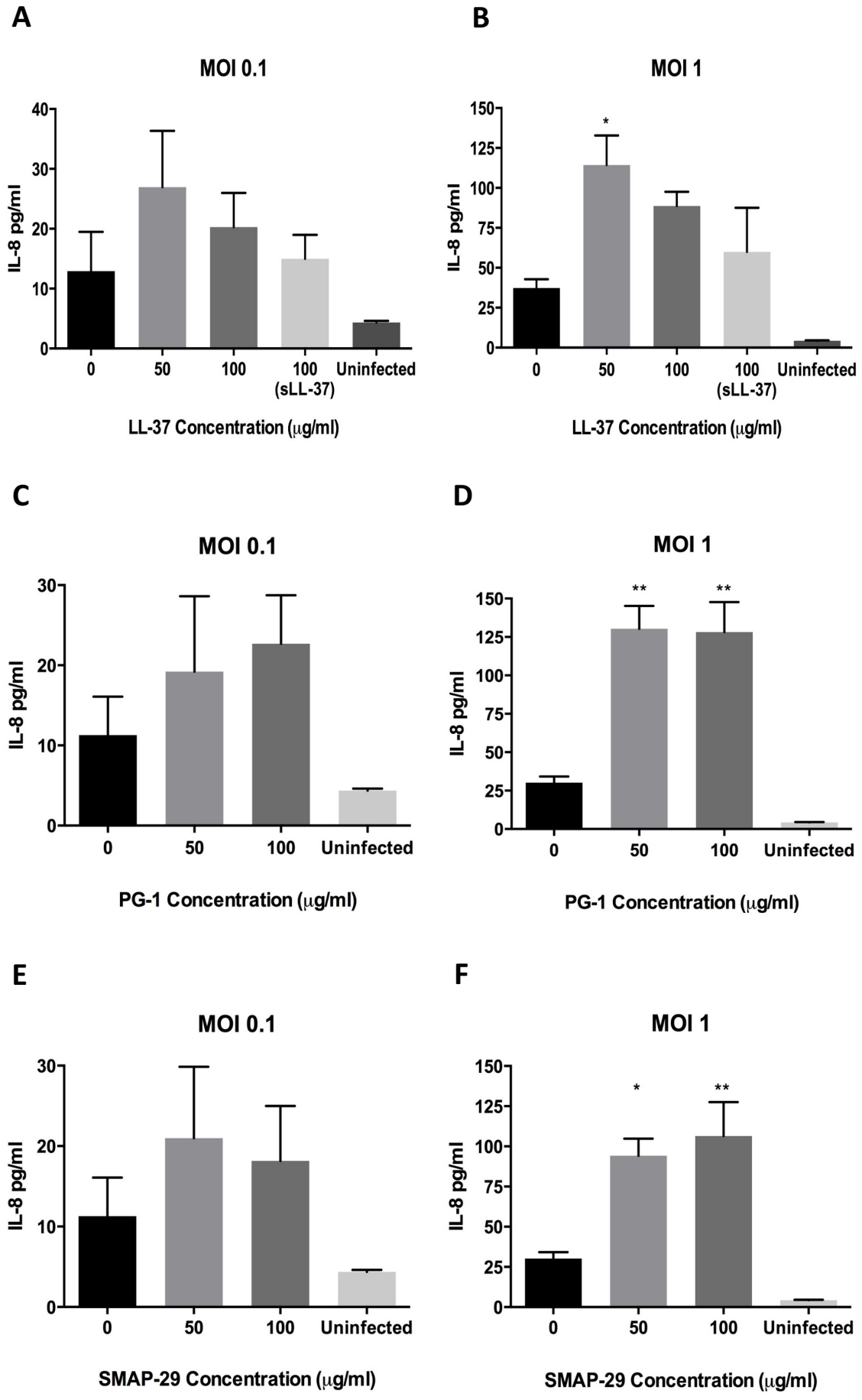

A
MOI 0.1
IL-8 pg/ml
40
30
20
10
0
0
50
100
100 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
B
MOI 1
IL-8 pg/ml
150
125
100
75
50
25
0
*
0
50
100
100 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
C
MOI 0.1
IL-8 pg/ml
30
20
10
0
0
50
100
Uninfected
PG-1 Concentration (μg/ml)
D
MOI 1
IL-8 pg/ml
150
125
100
75
50
25
0
**
**
0
50
100
Uninfected
PG-1 Concentration (μg/ml)
E
MOI 0.1
IL-8 pg/ml
30
20
10
0
0
50
100
Uninfected
SMAP-29 Concentration (μg/ml)
F
MOI 1
IL-8 pg/ml
150
125
100
75
50
25
0
*
**
0
50
100
Uninfected
SMAP-29 Concentration (μg/ml)

Figure 24. IL-8 release (pg/ml) after *W. chondrophila* exposure to cathelicidins. *W. chondrophila* (MOI 0.1 and 1) was exposed for 1 hour to different cathelicidin concentrations prior host cell infection for a further 1 hour. After 24 hours incubation, supernatants were collected and analysed by ELISA using uninfected cells as control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the infected control sample. *p≤0.05, ** p≤0.01.

3.4.3.2 Host cell exposure to LL-37, PG-1 and SMAP-29 prior to W. chondrophila infection does not alter IL-8 production

HEp2 cells were exposed to cathelicidins for 1 hour and then infected with *W. chondrophila* (MOI 0.1 and 1) for a further 1 h. Supernatants were recovered at 24 hours post infection and were analysed by ELISA. Cathelicidin pre-treatment of the host cell did not appear to mediate any alteration in the IL-8 response of the host cell to infection (Figure25A-25F). Host cell exposure to cathelicidins prior to infection resulted in a higher IL-8 released than when the bacteria was directly exposed to the peptides (Figure 24). Host cell pre-treatment did not result in a differential IL-8 production comparing to the untreated control, contrary to when the bacteria was exposed to different cathelicidin concentrations, which resulted in a higher IL-8 production than the infected control.

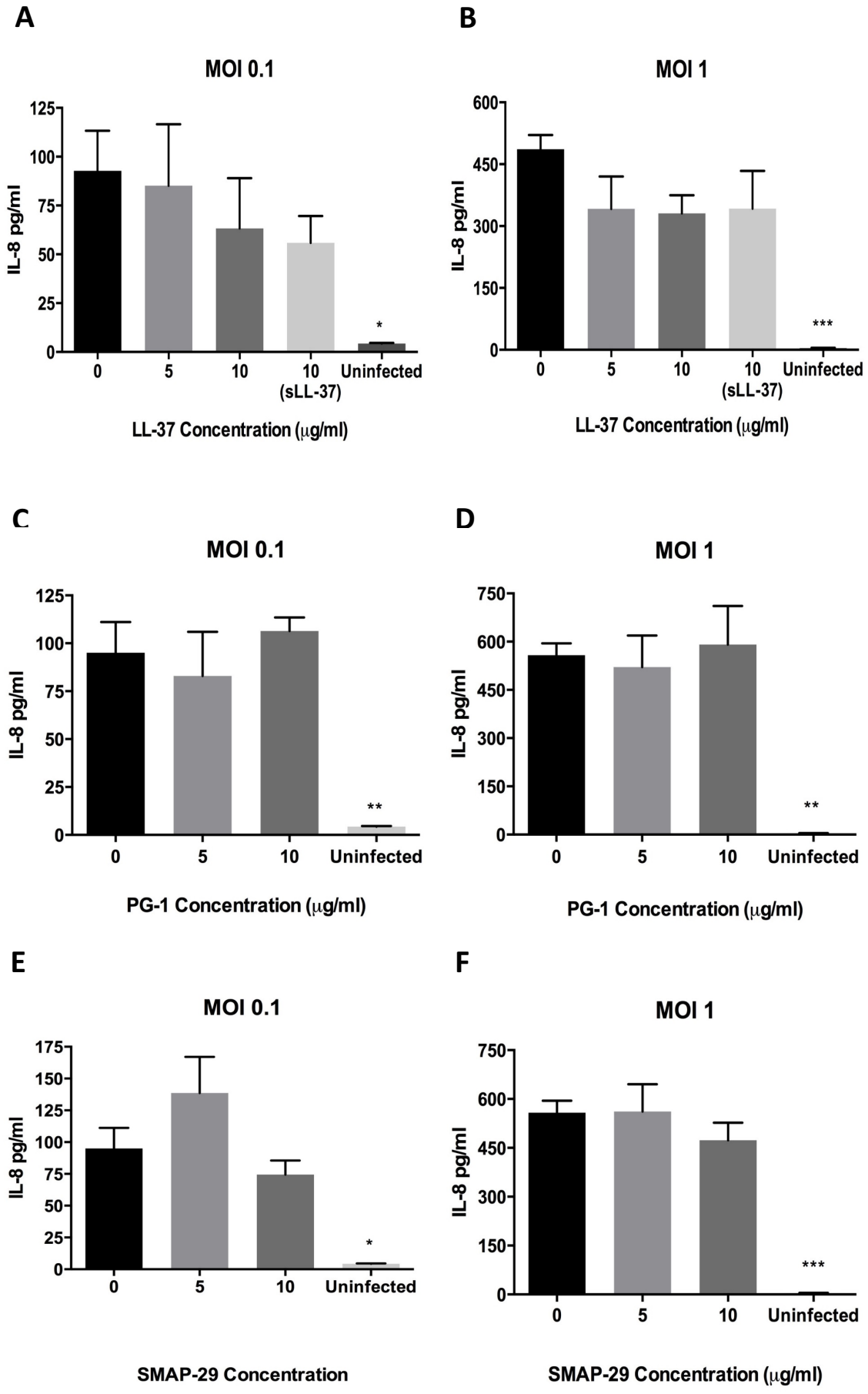

A
MOI 0.1
IL-8 pg/ml
125
100
75
50
25
0
*
0
5
10
10 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
B
MOI 1
IL-8 pg/ml
600
450
300
150
0
***
0
5
10
10 (sLL-37)
Uninfected
LL-37 Concentration (μg/ml)
C
MOI 0.1
IL-8 pg/ml
125
100
75
50
25
0
**
0
5
10
Uninfected
PG-1 Concentration (μg/ml)
D
MOI 1
IL-8 pg/ml
750
600
450
300
150
0
**
0
5
10
Uninfected
PG-1 Concentration (μg/ml)
E
MOI 0.1
IL-8 pg/ml
175
150
125
100
75
50
25
0
*
0
5
10
Uninfected
SMAP-29 Concentration
F
MOI 1
IL-8 pg/ml
750
600
450
300
150
0
***
0
5
10
Uninfected
SMAP-29 Concentration (μg/ml)

Figure 25. IL-8 release (pg/ml) after host cell exposure to cathelicidins prior to infection with *W. chondrophila*. HEp2 cells were incubated for 1 hour with various cathelicidin concentrations and then infected for a further 1 hour with *W. chondrophila* (MOI 0.1 and 1). After 24 hours incubation, supernatants were collected and analysed using infected cells without peptide treatment as a control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the infected control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.5 Intracellular localisation of Dansyl-labelled LL-37

3.5.1 dLL-37 can enter the host cell

To determine if LL-37 is internalised and retained by the host cell, HEp2 cells were exposed to dansyl-labelled-LL-37 for 1 hour, fixed and stained with mounting medium containing DAPI. Slides were imaged by confocal microscopy (Figure 26). The images confirmed the presence of LL-37 in the cytoplasm of the cell at this time point. To avoid conflicting detection due to overlapping of the excitation and emission spectrum of both fluorophores: dLL-37 λ_{ex} 338nm, λ_{em} 500nm, DAPI (λ_{ex} 360nm, λ_{em} 460nm), cells were also treated with dansyl-LL-37 and a mounting media without DAPI was used (). Imaging by confocal microscopy revealed an extra-nuclear presence of dLL-37 inside HEp2 cells.

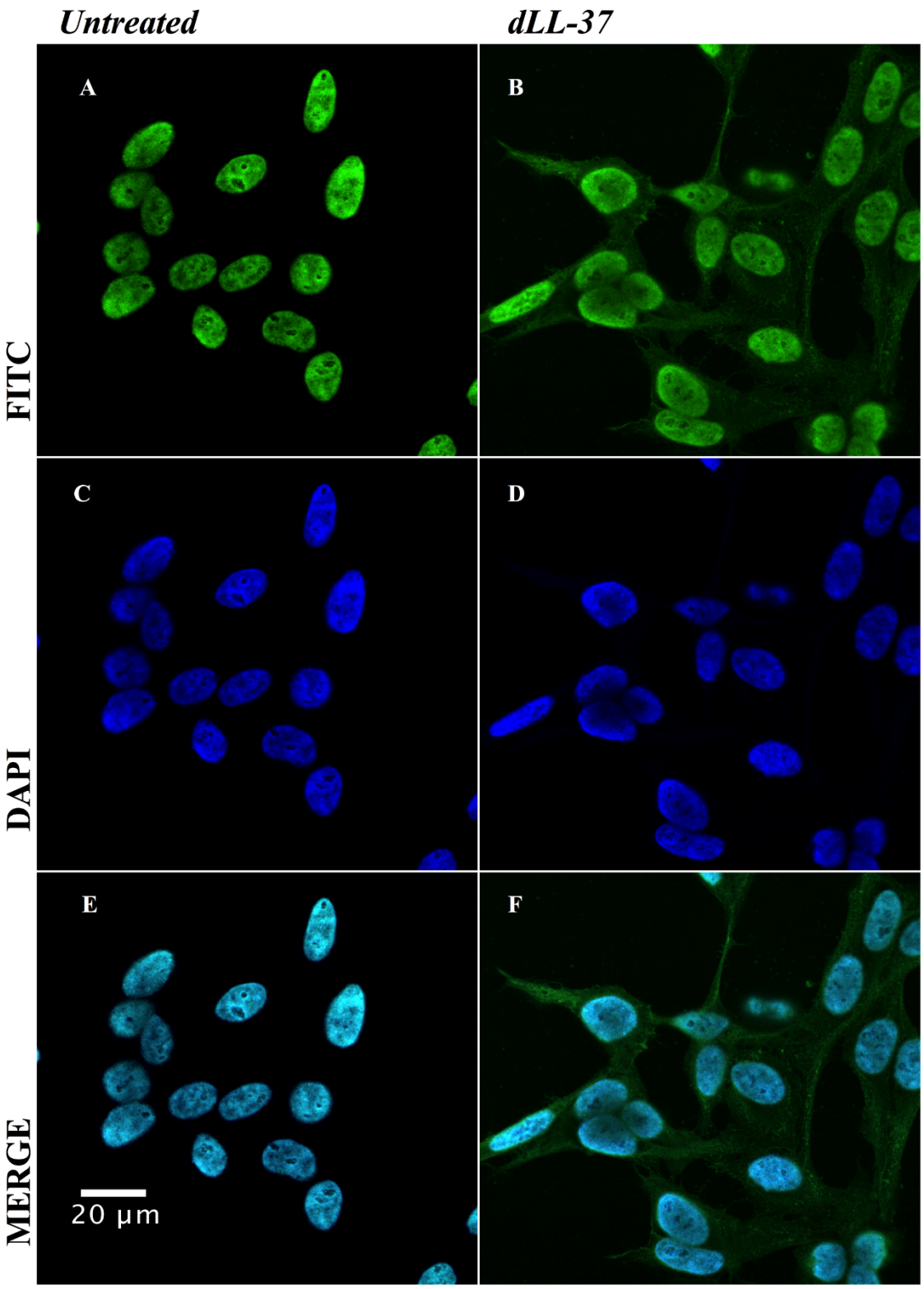


Figure 26. Dansyl-LL-37 localisation within HEp2 cells counterstained with DAPI. HEp2 cells were treated with dansyl-LL-37 (10µg/ml) for 1 hour at 37 ºC. Cells were fixed and DAPI stain was added before imaging by confocal microscopy. A merged DAPI/ dansyl-LL-37 (E, F) images are shown, nuclei in blue (C, D) and dansyl-labelled LL-37 in green (A, B).

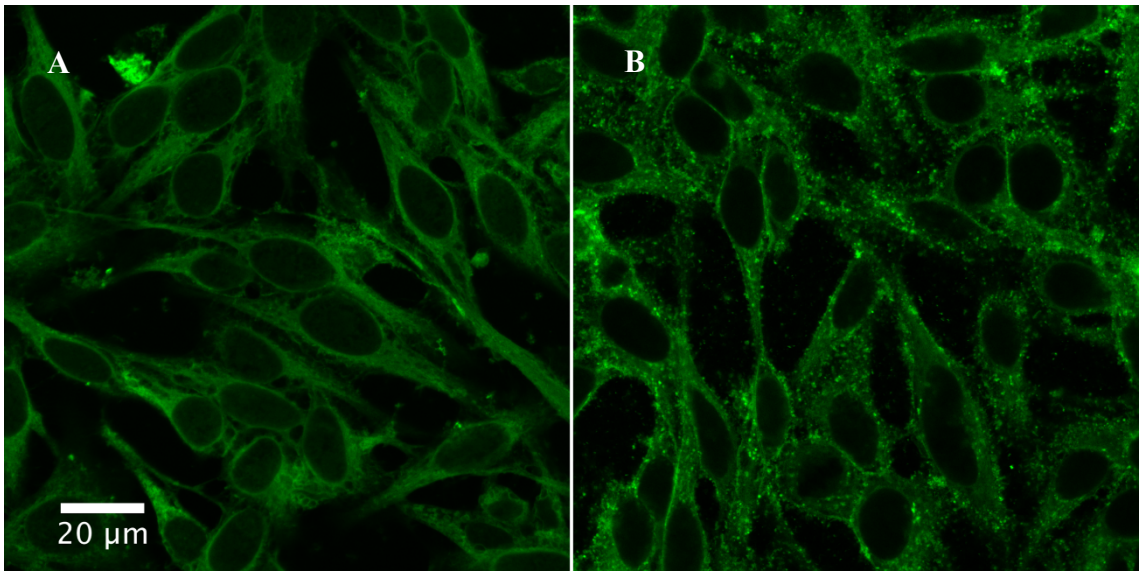


Figure 27. Dansyl-LL-37 localisation within HEp2 cells. HEp2 cells were treated with dansyl-LL-37, 10µg/ml (A) and 30µg/ml (B) for 1 hour at 37 ºC. Cells were fixed and the slides were mounted in mounting media without DAPI prior imaging by confocal microscopy.

3.5.2 Dansyl-LL-37 co-localizes with the intracellular bacterial inclusions

In order to determine if infection alters intracellular LL-37 localisation, HEp2 cells were infected for 1 h with *C. trachomatis* or *W. chondrophila.* (MOI 1). Media containing extracellular bacteria was then removed and fresh culture media was added. After a further 23 hours of incubation, cells were exposed to 10µg/ml of dansyl LL-37 for 1 hour. Cells were then washed and fixed with acetone prior to imaging by confocal microscopy. LL-37 appeared to colocalize with *Waddlia chondrophila* inclusions, as indicated by orange staining, but not with *C. trachomatis* inclusions (Figure 28).

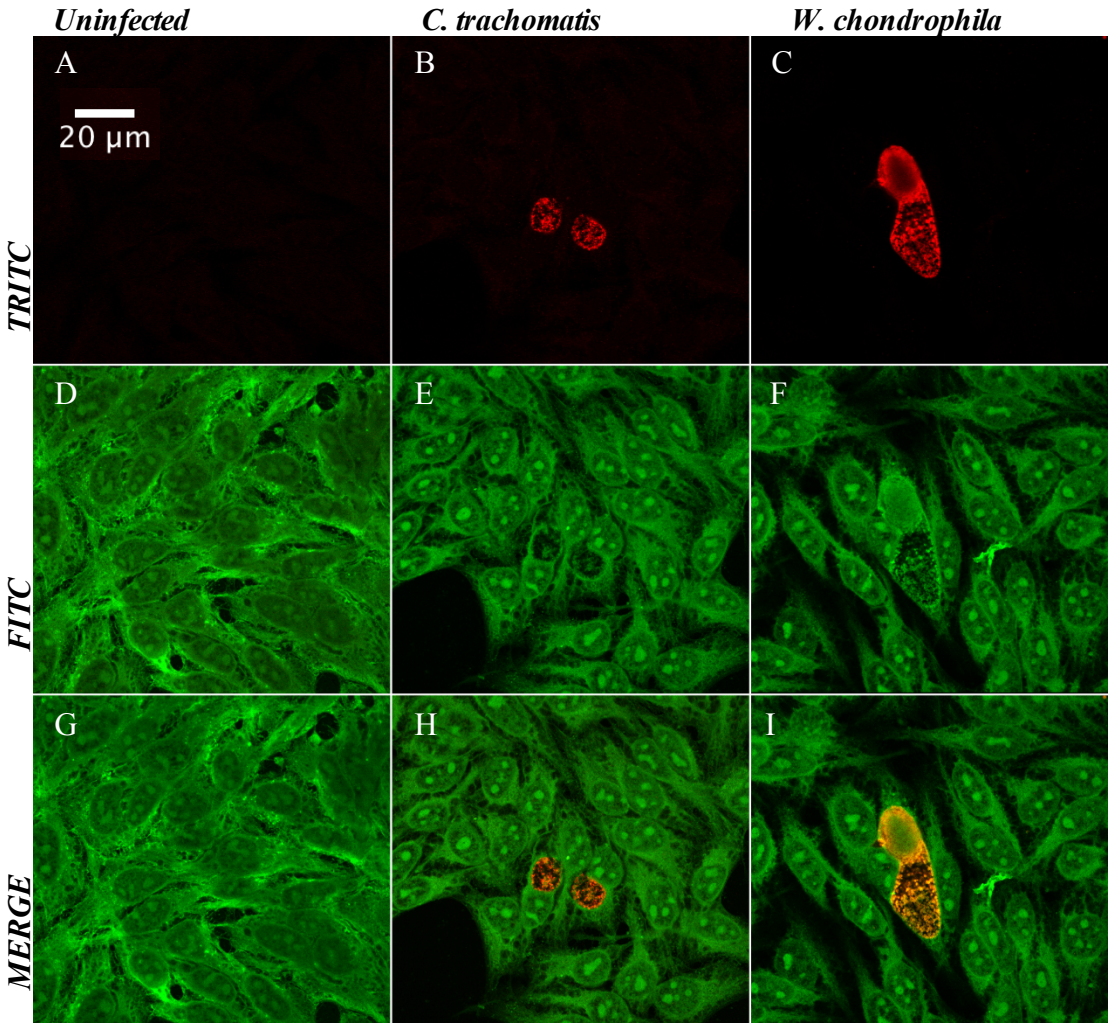


Figure 28. Dansyl-labelled-LL-37 treatment of HEp2 infected cells (G, H and I). HEp2 cells were treated with dansyl-LL-37, 10µg/ml for 1 hour at 37 ºC. Cells were fixed and the slides were mounted prior imaging by confocal microscopy. *C. trachomatis* and *W. chondrophila* are seen in red (B and C respectively), dLL-37 is seen in green (D, E and F).

4 Discussion

4.1 The direct antimicrobial effects of cathelicidins on C. trachomatis and W. chondrophila

Cathelicidins have demonstrated powerful antibacterial and antiviral activity as well as immunomodulatory properties (Kościuczuk *et al.*, 2012; Barlow *et al.*, 2014). Knowing the potential of cathelicidins against intracellular pathogens, we have chosen three cathelicidins: human LL-37, porcine PG-1 and ovine SMAP-29 for comparative analysis of their antimicrobial activity against *Waddlia chondrophila* and C*hlamydia trachomatis*. In this study, the direct antimicrobial effect of cathelicidins on *C. trachomatis* and *W. chondrophila* was studied. For both pathogens, changes in replication and infectivity were studied as endpoints to determine the protective and therapeutic efficacy of cathelicidins against these infections.

Only a limited number of studies have been published which have studied chlamydial responses to CHDP, with the primary focus being on cathelicidins and defensins (Yasin *et al.*, 1996; Chong-Cerrillo *et al.*, 2003; Donati *et al.*, 2005). Most of the studies that have been performed so far were focused on members of the Chlamydiaceae family, leaving other families of the Chlamydiae phylum behind. Very little is known about the effect of cathelicidins on *Waddlia chondrophila* infection. Coyle *et al.,* demonstrated that in response to *W. chondrophila* infection of ovine AH-1 trophoblasts, ovine defensins expression was upregulated, though no upregulation of the ovine cathelicidin SMAP-29 was observed, as only constitutive levels were found to be expressed (Coyle *et al.*, 2016). Donati *et al*. also studied the activity of different cathelicidins against *Simkania negevensis*, a Gram-negative bacterium belonging to the family of Simkaniaceae in the order Chlamydiales. *S. negevensis* was not found to be sensitive to LL-37, even at very high concentrations of the peptide (80µg/ml). On the contrary, it was found to be susceptible to PG-1 and SMAP-29 at very low concentrations (1 to 0.1µg/mL).

4.1.1 Exposure of Chlamydia trachomatis to LL-37, PG-1 or SMAP-29 prior to host cell infection reduces replication and infectivity

In this study, *Chlamydia trachomatis* L2 was directly exposed to cathelicidins prior to the addition of the pathogen to host cells, and pathogen replication was found to be affected. Replication was decreased after cathelicidin exposure, with SMAP-29 being the most effective peptide. The potent antimicrobial activity of SMAP-29 has been previously observed against various pathogens. In the study of different direct cathelicidin treatments against clinical strains of *P. aeruginosa,* SMAP-29 was found to be the most effective cathelicidin in preventing bacterial growth (Saiman *et al.*, 2001). SMAP-29 also resulted the most effective cathelicidin against other bacterial species, including *S. aureus* and *E. coli*, as it prevented its growth at lower peptide concentrations and showed a broad spectrum activity (Skerlavaj *et al.*, 1999).

Infectivity, as measured by a significant decrease in chlamydial inclusions, was also decreased by all cathelicidins used in this study, with 50μg/ml being the most effective concentration for all peptides (Figure 12). These results support the findings obtained in previous studies of *C. trachomatis* responses to cathelicidin exposure that resulted in infectivity reductions. Other studies have shown that when different *C. trachomatis* serovars were exposed to PG-1, the number of inclusion-forming units (IFU), which is directly equivalent to the number of inclusions, was highly reduced (Yasin *et al.*, 1996; Chong-Cerrillo *et al.*, 2003). This decrease was more potent than when *C. trachomatis* was exposed to defensins. L2 was found to be the most susceptible serovar to PG-1 and other tested antimicrobial peptides when compared to serovars E and H (Chong-Cerrillo *et al.*, 2003). The susceptibility to PG-1 was also noted by Donati e*t al.,* though this was at substantially higher concentrations (80μg/ml ± 1.4μg/ml). The most effective antimicrobial activity was found by when *C. trachomatis* was exposed to SMAP-29, as it was determined that there was a 50% drop in infectivity at a concentration of 10μg/ml (Donati *et al.*, 2005). Other *Chlamydia* species have shown more susceptibility to SMAP-29 than to PG-1 regarding infectivity. This is the case in the context of *Chlamydia suis* (Donati *et al.*, 2007) and *Chlamydia pneumoniae* exposure (Donati *et al.*, 2005).

Our findings confirmed an antimicrobial effect of cathelicidins in *C. trachomatis* infection models. However, differences between studies in the effectiveness of the

peptides may be attributed to the host cell model used in the experiments performed by Donati *et al.,* and Yasin *et al*., which were animal host cell models. In contrast, all the experimental work in our study used a human host cell model. Another important difference between previous studies and the methodology followed in this work is the composition of the solutions in which the peptides were dissolved. Host defence peptides are known to exhibit salt sensitivity to high salt conditions, such as those found in PBS, which can affect their antimicrobial activity. In addition, serum has also been shown to inhibit cathelicidin activity (Travis *et al.*, 2000; Dürr, Sudheendra and Ramamoorthy, 2006). With these considerations, this work was conducted using physiological conditions in all the experiments, whereby peptides and bacteria were diluted and incubated in serum-free host cell culture media (IMDM) to allow cathelicidins the best possible chance to exhibit inhibitory effects. This contrasts with the experimental approach employed by Donati *et al.,* who used stock peptide solutions made with PBS, further diluted in SPG buffer (composed of sodium phosphate, sucrose and glutamic acid) for the incubation with bacteria. Yasin *et al*., also used a similar buffer in their experiments.

The data obtained in this work points to cathelicidins exerting inhibitory effects in the context of both pathogen replication and in infectivity, although they appear to be more effective at altering infectivity. While cathelicidin treatment of *C. trachomatis* had an impact on host cell infection, it would be anticipated that the functional consequence of this would be that less elementary bodies would enter the host cell, differentiate and multiply. This is likely due to the direct antimicrobial effect as previously described, mediated by bacterial membrane permeabilisation and the consequent death of the organisms (Dawson and Liu, 2009; Bolintineanu and Kaznessis, 2011).

4.1.2 Exposure of W. chondrophila to LL-37, PG-1 or SMAP-29 increases replication and infectivity

In contrast to the inhibition of infectivity and replication of *C. trachomatis* by cathelicidins, direct exposure of *W. chondrophila* to cathelicidins prior to the addition of the pathogen to host cells induced an increase in replication and infectivity. The lowest concentrations of LL-37 and PG-1 (10μg/ml) were sufficient to observe significant

increases in *Waddlia* infectivity (Figure 13). As *W. chondrophila* has only relatively recently been characterised, there is a paucity of research in terms of its susceptibility to antimicrobial treatments, in particular HDPs. Our results suggest that cathelicidins have species specific effects towards chlamydial pathogens, and that *W. chondrophila* does not demonstrate any sensitivity to these peptides. This observation was relatively unexpected, as resistance to antimicrobial peptides has only been previously described in certain Gram-negative bacteria. For example, *Staphylococcus aureus* can secrete a protein (Staphylokinase) that can interact with and neutralize α-defensins (Jin *et al.*, 2004), and *E. coli* can produce an outer membrane protease, OmpT that can protect the bacteria by mediating the proteolytic degradation of LL-37 (Thomassin *et al.*, 2012). In mCRAMP (LL-37 orthologue) knockout mice, *E. coli* was found to be less infective than in wild-type mice (Danka and Hunstad, 2015), which holds some similarity with what has been observed with *Waddlia* in this study.

Other resistance mechanisms employed by bacteria involve net cell surface charge alteration, peptide efflux from the bacterial cytoplasm to the extracellular space, restriction of antimicrobial peptide access to their targets and proteolytic cleavage of the peptides (Cole and Nizet, 2016). *W. chondrophila* is not only resistant to the direct antimicrobial activity of all cathelicidins tested, but also appears to thrive in its presence. In contrast to what was observed with *W. chondrophila*, cathelicidin peptides have shown substantial *in vitro* activity against *C. trachomatis*, *C. pneumoniae* and *Simkania nevegensis*. However, some animal Chlamydiae, including *Chlamydia abortus*, *Chlamydia psitacci* and *Chlamydia felis*, were not particularly sensitive to cathelicidins even at very high concentrations (>80μg/ml)(Donati *et al.*, 2005) although this may be attributable to the experimental conditions as previously discussed.

In the context of the involved mechanisms, charge may play a role. The three peptides used in this study have different net charges, with SMAP-29 displaying the most cationic (net charge +10) and LL-37 and PG-1 less cationic (both with net charge +6). Increased cationic charge is associated with increased antimicrobial activity against Gram-negative and Gram-positive bacteria (Travis *et al.*, 2000; Sorensen and Borregaard, 2005; Dawson and Liu, 2009). We would suggest that other mechanisms must be involved, as the data obtained from cathelicidin treatment of *C. trachomatis* shows that not only SMAP-29 is one of the most effective reducing replication and infectivity, but also PG-1. Data

obtained from LL-37 and PG-1 treatments of *W. chondrophila* indicates more substantial increases in replication and infectivity in response to these peptide treatments, which joined to *C. trachomatis* results, could suggest that the charge of this peptides might not be key for the peptide effect on these pathogens. It may be possible that other peptide variables, such as peptide sequences, might play a role.

4.2 The effect of host cell exposure to cathelicidins on C. trachomatis and W. chondrophila infection

In addition to the direct antimicrobial effect of cathelicidins, we also examined the potential for cathelicidins to alter the host pathogen response by direct treatment of the host cell. In this context, host cells were exposed to the peptides prior to infection and effect on bacterial infectivity and replication was then assessed as before. The cathelicidin concentrations used in this set of experiments were identical to the peptide concentrations that the host cells were exposed to after direct pathogen-peptide exposure.

4.2.1 Host cell exposure to LL-37, but not PG-1 or SMAP-29, alters subsequent C. trachomatis replication

When host cells were exposed to cathelicidins prior to infection, *C. trachomatis* infectivity was unaltered (Figure 15). In contrast, host cell pre-treatment with LL-37 apparently reduced *C. trachomatis* replication due to a decrease in the copy number detected by qPCR (Figure 14). Most studies performed with cathelicidins have focused upon the direct antimicrobial properties of the peptides against different pathogens, and host cell exposure to peptides have been studied to much a lower extent, though the immunomodulatory role of cathelicidins is well known. LL-37 can induce the production of several chemokines, though there is some debate as to whether this is achieved by direct chemotaxis induction or it happens indirectly by induction of classical chemokines (Shafer, 2006). LL-37 can up-regulate receptors which a broad range of ligands such as chemokines, interleukins and hormones. These effects displayed by LL-37 may contribute to the protective effect that we observed after LL-37 treatment of host cells prior to *C. trachomatis* infection. Again, the fact that the host cell line used in all our

studies was human derived may be one of the factors to explain why LL-37, the sole human cathelicidin, was particularly effective in reducing bacterial replication. The observation that replication and not infectivity has been altered also support the hypothesis that pathogen adhesion and entry is not altered by host cell pre-treatment.

Previous studies have also found that cathelicidins might hold potential as prophylactic treatments against infection. For example, in an animal model, Schneider *et al*., showed that prophylactic treatment of zebrafish embryos with the chicken cathelicidin, CATH-2, led to a partial protection against lethal bacterial infections, such as those caused by *Salmonella* (Schneider *et al.*, 2016). In models of hepatitis C viral infection, LL-37 was also observed to have a key protective effect as host cells pre-treatment with LL-37 attenuated virus infectivity (Matsumura *et al.*, 2016). Other studies have aimed to investigate if cathelicidin pre-treatment of host cells could trigger host cell responses that would lead to differential effects post infection.

In our study, the mechanisms involved in the LL-37 mediated reduction in bacterial replication are not fully understood. We hypothesised that cathelicidin treatment could provoke an inflammatory state in the host cells which would attenuate *C. trachomatis* infection. This should therefore be the subject of additional work to perform a full characterization of the processes involved.

4.2.2 Host cell exposure to LL-37, PG-1 or SMAP-29 does not alter W. chondrophila replication and infectivity

In our study, when host cells were exposed to cathelicidins prior to infection, *W. chondrophila* infectivity and replication were unaltered. This suggests that any host-cell based mechanism by which LL-37 reduced *C. trachomatis* replication was not effective in *W. chondrophila*. However, in contrast to our observations with *C. trachomatis*, *W. chondrophila* was resistant to the antimicrobial properties of the peptides, and this could therefore be a factor in this observation. We could hypothesize that a direct interaction occurs between peptide and bacteria that favours enhanced bacterial adhesion and internalization, although this requires further experimental study.

In addition, differences between this two pathogens could be key in their opposing responses to host-cell treatment with the peptide. The chlamydial outer membrane, which is crucial for adhesion and invasion, is comprised of several different proteins between species. Many proteins of the outer membrane complex (OMC) of the Chlamydiaceae family, such as porin B and polymorphic outer membrane proteins (Pmps), have no homologues in *Waddlia*. Further studies could focus upon elucidating a role for these proteins in susceptibility to cathelicidin exposure.

Furthermore, despite growing in a variety of human cell lines, *W. chondrophila* lacks some features associated with *C. trachomatis* infection, such as inhibition of apoptosis (Dille *et al.*, 2015). Given the established relationship between host defence peptides and modulation of cell death pathways, this was an area of interest for us in terms of elucidating the exact underlying mechanisms in our observations and is discussed below.

4.3 The effect of cathelicidins exposure on host cell death

The direct antimicrobial activity of cathelicidins has been shown to involve interactions with the pathogen membrane and subsequent permeabilisation (Dawson and Liu, 2009; Bolintineanu and Kaznessis, 2011). It has been observed that CHDP preferentially permeabilize prokaryotic membranes, although the mechanisms of membrane discrimination, between the pathogen and the host cell membranes, is still not fully understood (Chen *et al.*, 2007). Chen *et al.,* suggested that peptide specificity between eukaryotic and prokaryotic cells depends upon the compositional difference in the lipids between the two types of membranes.

It has been well established that high concentrations of cathelicidins can promote host cell death by necrosis. SMAP-29 has been found to be the most cytotoxic compared to other ovine cathelicidins used, reducing the viability of a number of different cell lines by 50% from 50-100µg/ml concentrations (Jacob *et al.*, 2014). Similarly, the *in vitro* cytotoxicity of LL-37 was studied in A549 cells and concentrations lower than 50µg/ml of the peptide were found not induce to host cell death (Gordon *et al.*, 2005). A strong

relationship between dose- and time-dependent cytotoxic activity was found in HepG2 cells after PG-1 exposure (Niu *et al.*, 2015).

Although the cytotoxicity of cathelicidins is variable among host cell lines and depends upon the dosage and time of exposure, the concentrations used in our experiments did not promote host cell death. These results confirmed our predictions about using a safe range of cathelicidins that will not promote host cell death but will have an impact on the pathogens. To establish if the peptide concentrations used in this study promoted host (HEp2) cell death during infection, cytosolic lactate dehydrogenase release (LDH) was studied as an indicator of plasma membrane disruption. It was found that, when cells were exposed for 1 hour to both cathelicidins and bacteria, there was no significant increase in LDH release into the supernatant in comparison to the untreated controls (Figure 18 and 19). Infection with *Waddlia chondrophila* alone also did not lead to an increased LDH release increase. However, in *C. trachomatis* infection and SMAP-29 treatment, the LDH release was higher than the uninfected control though the percentage of total LDH release was still relatively low (≤15% total LDH release). Given that SMAP-29 has previously been shown to be one of the most cytotoxic cathelicidins in the context of mammalian cells, this observation is interesting as it indicates that induction of host cell death is unlikely to be the mechanism for reductions observed in bacterial replication.

Previous studies have observed increased LDH release and cytotoxicity mediated by cathelicidins in the context of other pathogens. Sousa *et al.,* observed a reduction in host cell metabolic activity during HRV infection and exposure to ≥20μg/ml LL-37, although there was not significant increase in LDH release at the concentrations of LL-37 tested. However, in healthy cells, exposure to a superphysiological concentration (100μg/ml) of LL-37 for 6 hours did induce a significant increase in LDH released from cells, suggesting a time-dependent cytotoxic effect only at the highest concentration (Sousa *et al.*, 2017). Notably, in influenza A virus-infected neutrophils that were exposed to LL-37, a significant increase in LDH release was not observed at peptide concentrations lower than 15μg/ml (Tripathi *et al.*, 2014). In the transformed bronchial epithelial cell line, 16HBE14^{o-} the induction of host cell death by apoptosis after LL-37 treatment and *P. aeruginosa* infection was studied (Barlow *et al.*, 2010). It was shown that only when both bacteria and peptide were added to the cells, apoptosis was significantly increased at lower peptide concentrations.

In summary, our results indicate that the cathelicidin concentrations used in this study were not cytotoxic towards HEp2 cells via the induction of necrosis at the time points studied. This would suggest that induction of host necrotic cell death by cathelicidins was not a mechanism by which LL-37, or indeed the other cathelicidins used, were exerting indirect antimicrobial effects towards *C. trachomatis*.

4.4 The effect of cathelicidins on the host cell inflammatory response during C. trachomatis and W. chondrophila infection

Disease and pathology caused by *Chlamydia* infection can be a result of sustained inflammation-associated tissue damage (Beatty, Byrne and Morrison, 1994; Darville and Hiltke, 2010). The pro-inflammatory cytokine, Interleukin-8 (IL-8), has been shown to be produced by cells in response to *C. trachomatis* infection (Rasmussen *et al.*, 1997).
Due to the broad immunomodulatory activities of cathelicidins (Turner *et al.*, 1998; Kościuczuk *et al.*, 2012; Barlow *et al.*, 2014), we studied the inflammomodulatory effect of the peptides in the host cell response to chlamydial infection. To elucidate if the peptides alone were sufficient to elicit an inflammatory response, HEp2 cells were treated with cathelicidins and IL-8 release by these cells was quantified. Our data revealed that the IL-8 release by these cells in response to cathelicidin treatment was relatively low (<40pg/ml) and was not significantly different from the untreated control (Figure 20).

Previous work in other cell lines, such as keratinocytes, has revealed that LL-37 promoted IL-8 production at concentrations higher than 13μg/ml (Braff *et al.*, 2005), in neutrophils in a dose- and time-dependent manner at concentrations between 10-40μg/ml (Zheng *et al.*, 2007) and in human airway smooth muscle cells at 10μg/ml (Zuyderduyn *et al.*, 2006). The effect of LL-37 on chemokine induction in the human epithelial cell line A549 was studied confirming the ability of this peptide to promote a significant increase in the IL-8 production at concentrations higher than 20μg/ml (Scott *et al.*, 2002; Mookherjee *et al.*, 2006).

4.4.1 Cathelicidins enhance IL-8 release by host cells during C. trachomatis infection

C. trachomatis was exposed to a range of cathelicidin concentrations, and the exposed bacteria subsequently used to infect HEp2 cells. IL-8 release from the cells was measured by ELISA. It was found that exposure of *C. trachomatis* to cathelicidins prior to infection dramatically enhanced IL-8 release by the host cell. This was observed 48h after infection, and LL-37 was found to be most effective at altering IL-8 release (Figure 21) compared to the other peptides. It has been shown that IL-8 is produced by *C. trachomatis* infected epithelial cells in a bacterial growth-dependent manner (Buchholz and Stephens, 2006). In this model, we observed that the enhanced IL-8 release took place in the context of reduced bacterial infectivity and a reduction in the number of *C. trachomatis* infected cells.

Dose dependent IL-8 release induced by cathelicidin treatment and in the presence of bacterial components, such as LPS, has been previously studied in neutrophils. When LPS was exposed to cell in the presence of LL-37, it was found that IL-8 release was decreased (Alalwani *et al.*, 2010). The same effect was also observed when whole inactivated Gram- and Gram+ bacteria were used. In the epithelial line, A549 the same observation was recorded, confirming a role of LL-37 in reducing bacterial stimulation of inflammatory mediators (Scott *et al.*, 2002). Interestingly, Scott *et al.* also observed that at higher concentrations of LL-37 (50–100μg/ml), the stimulation of IL-8 production independent of LPS occurred. Concentrations below 50μg/ml were shown to bind to bacterial components, like LPS, preventing its interaction with host cell receptors, such as TLR4, and reducing the inflammatory response (Scott *et al.*, 2000, 2002).

In our study, when host cells were exposed to cathelicidins prior to infection, IL-8 release was not altered compared to the negative control. This may be due to the low, physiologically relevant concentrations of cathelicidins used in this study. It would be interesting to test higher, superphysiological peptide concentrations (>30μg/ml) to assess IL-8 release in HEp2 cells and compare them to cell lines tested in other published studies. PG-1 treatment resulted in a decrease in the IL-8 release, but again, it would be interesting to see the IL-8 production at higher PG-1 concentrations.

Furthermore, we note that our studies have only focused on one cytokine, but we must consider the role of other cytokines in the inflammatory response to infection. *Chlamydia* infection induces the secretion of interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, IL-18 and tumour necrosis factor alpha (TNF-α), among others (Rasmussen *et al.*, 1997; Gervassi *et al.*, 2004). Thus, to understand how the inflammatory response is being regulated by cathelicidins, it would be necessary to study the expression of other cytokines in the future. For example, IL-1α would be an ideal candidate as it is well known how this cytokine contributes to chlamydial induction of IL-8 production (Cheng *et al.*, 2008). Cheng *et al.,* found that cells expressing IL-1α, like HEp2 cells, significantly increased their production of IL-1α, IL-6, and IL-8 proteins after chlamydial infection. When the IL-1α receptor was blocked and the IL-1α was neutralised by antibodies, no decrease in the IL-8 production was seen at early stages and only a partial reduction was seen in later stages. That supports the hypothesis that different pathways independent of IL-1α must exists. Other studies also characterised the ability of IL-1 to induce IL-8 production by the host cell. In fallopian tube samples, it was observed that the use of an IL-1 receptor antagonist could avoid destructive effects of *C. trachomatis* infection by reducing the host cell inflammatory response (Hvid *et al.*, 2007).

Again, it is necessary to consider the variability among different cell lines and peptide dosage. It is possible that if we had used higher peptide concentrations in our studies we would have observed significant IL-8 production by HEp2 as had been previously shown in other cell lines. In the context of our study, increased IL-8 release in the presence of cathelicidins could be a result of enhanced PAMP release, due to pathogen death, and recognition by the host cells, although this would require further investigation.

4.4.2 Host cell IL-8 production is only altered after W. chondrophila exposure to LL-37, PG-1 and SMAP-29

The *Waddlia chondrophila* infective cycle is shorter than *C. trachomatis*. In contrast to *C. trachomatis*, all direct peptide treatments of *W. chondrophila* prior to infection resulted in significant increases in IL-8 production. The most significant effect was seen with use of the highest bacterial load (MOI 1). This may therefore be linked to the increased growth of the pathogen that can elicit a higher IL-8 release, which was comparable with

results previously shown by Wheelhouse *et al.* (Wheelhouse *et al.*, 2014). The same pattern is seen in our study when *C. trachomatis* was directly exposed to peptides, although both pathogens differed substantially regarding replication and infectivity. We previously noted that peptide and bacteria incubation may have induced bacterial death, potentially releasing bacterial PAMPs that could increase the inflammatory response in *C. trachomatis*. However, the opposing response in *W. chondrophila* suggests that other variables are implicated.

We would suggest that the increase in the pathogen number due to the peptide treatment could be promoting an enhanced inflammatory response. This hypothesis is supported by other studies which have observed that IL-8 is significantly increased in late stages of chlamydial infection and when the pathogen numbers were higher (Rasmussen *et al.*, 1997; Buchholz and Stephens, 2006; Cheng *et al.*, 2008).
In our study, host cell exposure to cathelicidin peptides did not produce an altered IL-8 response when compared to the untreated control. In addition, when host cells were pre-treated with peptides, infectivity and replication was not affected in *W. chondrophila* infection. While higher peptide concentrations may influence this, it must be considered how very high concentrations of the peptides might be toxic. Assessing how cathelicidins modulate the expression of other key cytokines and the possible impact on host cell membrane receptors is also a key area which requires further work. For this purpose, it might be necessary to consider other host cells, as HEp2 cells are known to lack functional TLR4 receptors on the membranes. As the lack of functional TLR4 does not affect *Chlamydia* adhesion and entry, this supports HEp2 cells as an adequate model for all the experiments performed in this work. However, comparable studies in other cells with functional TLR responses would help elucidate the mechanisms underlying the inflammatory response to this pathogen.

As it has been mentioned previously, chlamydial infection induces the secretion of several cytokines by the host cell, including IL-1. This cytokine can increase IL-8 production during late stages of chlamydial infection. However, the role of IL-1α has not been as extensively studied in *Waddlia* as in *Chlamydia* infection. The comparative study of the expression of this cytokine in both pathogens might show a differential response. When the ovine trophoblast cell line AH-1 was infected with *Waddlia,* an analysis of the subsequent cytokine production was performed, including assessment of IL-8 and IL-1

(Coyle *et al.*, 2016). An increase in the IL-8 response and in IL-1β was found, although the IL-8 increase was much more substantial. In contrast, IL-1α production was not increased significantly, even at a very high MOI (10).

4.5 Intracellular localisation of Dansyl-labelled LL-37

Our results indicated that cathelicidins mediated differential effects on *C. trachomatis* and *W. chondrophila* infectivity and replication, and thus we examined entry and localisation of the peptides in the host cell. Host cells were grown and infected with bacteria for 24 hours, and subsequently treated with fluorescently labelled LL-37. We observed that when healthy cells were treated with dansyl-labelled LL-37 for 1 hour, a predominantly cytoplasmic distribution in the host cells was observed. Interestingly, when cells were infected with the two pathogens and subsequently treated with dansyl-labelled LL-37 for one hour, peptide distribution within the cell appeared to be altered, with more nuclear distribution compared to the uninfected control. Dansyl-LL-37 localisation without infection (Figure 27) and the uninfected controls of dansyl-LL-37 treatment (Figure 28) should exhibit similar peptide distributions, as the treatment was the same. The fact that they exhibit differential peptide distributions might point that the Figure 27 immunostaining might be artefactual. We also observed that some colocalisation of dansyl-LL-37 and bacteria occurred in *Waddlia chondrophila* infection. We did not observe colocalisation between *C. trachomatis* and dansyl-LL-37, and this may be due to the life cycle stage, which might make necessary to repeat the experiment with a longer *Chlamydia* incubation. Our data provides tentative indications that the peptide could be colocalising with *Waddlia* inside the host cell, but this represents a potential avenue for further investigation.

Several studies have also looked at cathelicidin distribution in the host cell during infection. After 24 hours *E. coli* infection, Braff *et al*. found colocalisation between endogenous LL-37 and *E. coli,* thus pointing to the potential contribution of keratinocytes to cutaneous defence. In other studies utilising LL-37 immunostaining, it was shown that macrophages surrounded by high levels of LL-37 at infectious or inflammatory sites can

internalize cathelicidin to acquire higher bacterial killing activity (Tang *et al.*, 2015). This study also found that the internalized LL-37 phagocytosed bacteria colocalised in endosomal and lysosomal compartments, which suggested that LL-37 may target and eliminate pathogens directly in these compartments. In our studies, the treatment regimen involved the addition of LL-37 after 24 hours of infection, whereas in other studies it has been added prior to infection. While our approach examined cathelicidin localisation in an established infection, alternative treatment approaches would also be of value.

4.6 Final conclusions and future work

W. chondrophila has been utilised as a comparative model organism for the study of *Chlamydia* infection (Fehr *et al.*, 2016). However, its faster life cycle and its broad range of hosts as well as its higher virulence has highlighted differences from other organisms in the same class that would argue against its use as a model. Within this body of work, our data supports the concept that *W. chondrophila* holds very pronounced differences with *C. trachomatis* in the context of the host innate immune response. Our comparative studies with *C. trachomatis* L2 have indicated that its potential as a *Chlamydia* infection model is limited and that additional considerations must be made in future comparative studies.

Our data show that cathelicidins can interact with *W. chondrophila* and *C. trachomatis* in distinct ways. With *C. trachomatis* L2, we observed the classical and extensively described antimicrobial properties of cathelicidins against this pathogen, affirming that a therapeutic potential exists for cathelicidins to treat *C. trachomatis*. However, a significant body of work remains to be undertaken, regarding other chlamydial species and *in vivo* models of infection.

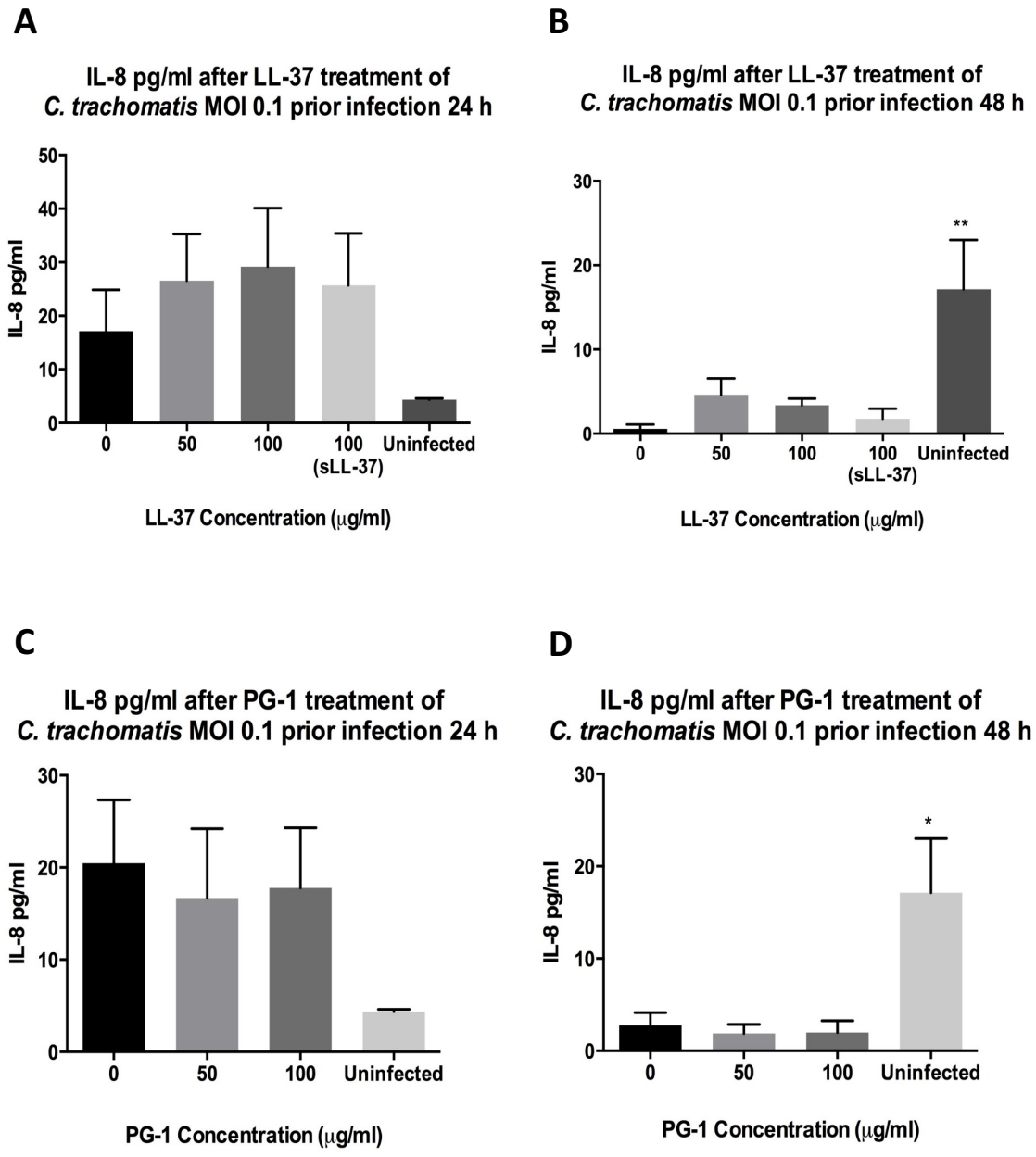
A limitation of the technique used to monitor bacterial replication (quantitative PCR) is that both the DNA of dead and viable bacteria is quantified. Future work would involve the use of more specific techniques, such as the Viability PCR, that could differentiate and quantify viable bacteria, the ones that would have the ability to infect after the treatments.

The novel results obtained with *W. chondrophila* point to an altered susceptibility to cathelicidin treatment and indeed, a specific interaction with these peptides. We observed that this pathogen may possess new mechanisms of resistance to these peptides and thus more work needs to be performed to clarify the nature of this interaction. In the context of inflammatory modulation by cathelicidins in *Waddlia* and *Chlamydi*a infection, we did not observe differential effects when comparing the two pathogens. However, we propose that to fully understand the potential of these peptides in modulating the inflammatory response, it will be necessary to study other key cytokines involved in the host response to chlamydial infection. Our work proves that although the two pathogens share a similar infection biology, they possess very different susceptibilities to cathelicidin treatments. Thus, a further investigation of these differences will not only provide a greater understanding of the underlying mechanisms of infection of these organisms, but inform the discovery and development of new treatments.

5 Results appendix

5.1 The effect of cathelicidins on IL-8 production in C. trachomatis infection

5.1.1 Direct cathelicidin treatment of C. trachomatis MOI 0.1



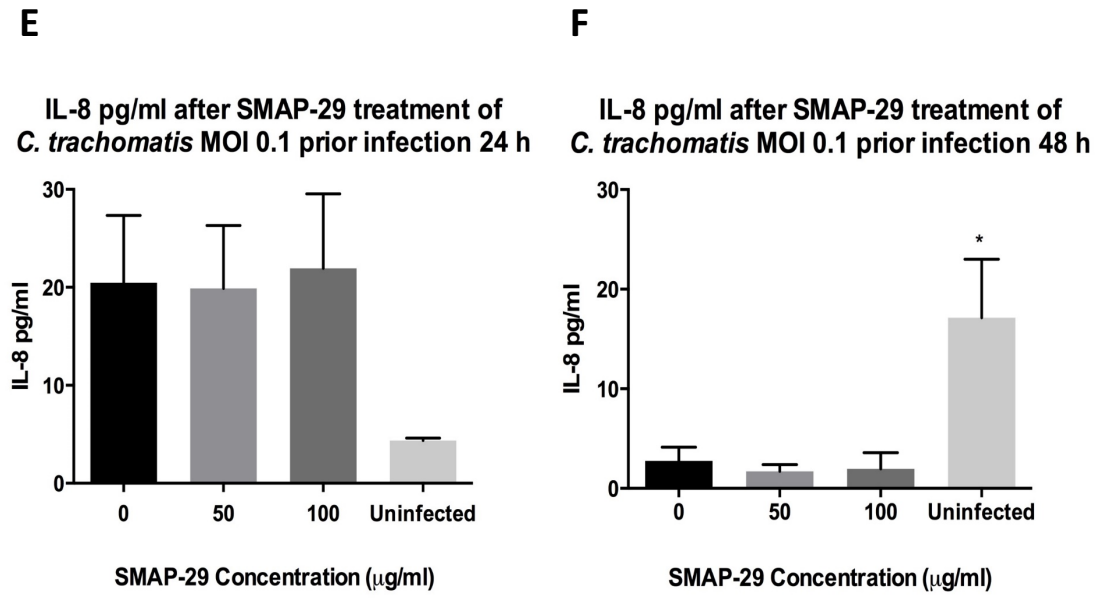
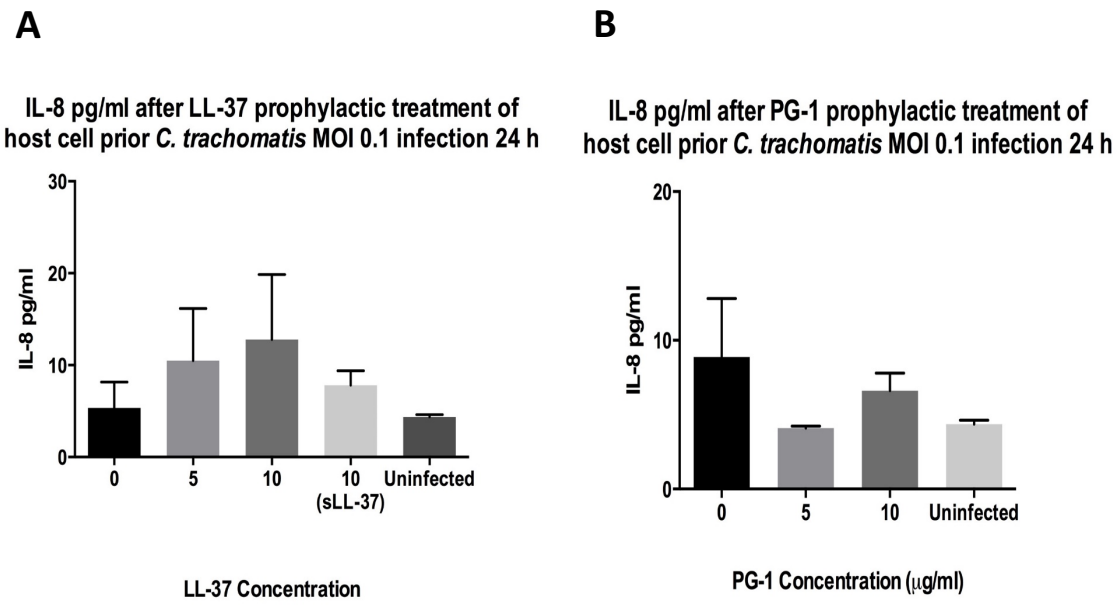


Figure 29. IL-8 release (pg/ml) after *C. trachomatis* exposure to cathelicidins. *C. trachomatis* MOI 0.1 was exposed for 1 hour to different cathelicidin concentrations prior host cell infection for a further 1 hour. After 24 hours and 48 hours incubation, supernatants were collected and analysed by ELISA using uninfected cells as control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the untreated infected control (0μg/ml). *p≤0.05, ** p≤0.01.

5.1.2 Host cell exposure to cathelicidins prior to C. trachomatis infection MOI 0.1



C

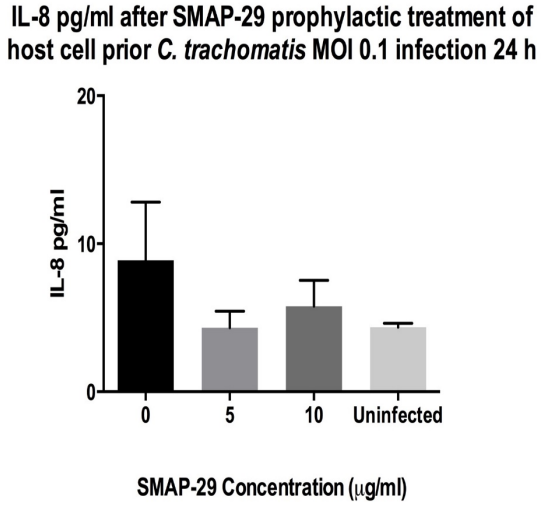


Figure 30. IL-8 release (pg/ml) after host cell exposure to cathelicidins prior to infection (MOI 0.1). HEp2 cells were pre-incubated for 1 hour with various cathelicidin concentrations and then infected for a further 1 hour with *C. trachomatis* (MOI 0.1). After 24 hours incubation, supernatants were collected and analysed using uninfected cells as control. Results are shown as a mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's *post-hoc* test to compare each treatment to the infected control (0μg/ml).

6 References

Agerberth, B. *et al.* (1991) 'Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides.', *European journal of biochemistry*, 202(3), pp. 849–54.

Alalwani, S. M. *et al.* (2010) 'The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils.', *European journal of immunology*. NIH Public Access, 40(4), pp. 1118–26. doi: 10.1002/eji.200939275.

Bachmann, N. L., Polkinghorne, A. and Timms, P. (2014) 'Chlamydia genomics: providing novel insights into chlamydial biology', *Trends in Microbiology*, 22(8), pp. 464–472. doi: 10.1016/j.tim.2014.04.013.

Barlow, P. G. *et al.* (2006) 'The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system', *Journal of Leukocyte Biology*, 80(3), pp. 509–520. doi: 10.1189/jlb.1005560.

Barlow, P. G. *et al.* (2010) 'The Human Cathelicidin LL-37 Preferentially Promotes Apoptosis of Infected Airway Epithelium', *American Journal of Respiratory Cell and Molecular Biology*. American Thoracic Society, 43(6), pp. 692–702. doi: 10.1165/rcmb.2009-0250OC.

Barlow, P. G. *et al.* (2014) 'Antiviral potential of cathelicidins', *Future Microbiology*, 9(1), pp. 55–73. doi: 10.2217/fmb.13.135.

Baud, D. *et al.* (2007) '*Waddlia chondrophila* , a Potential Agent of Human Fetal Death', *Emerging Infectious Diseases*, 13(8), pp. 1239–1243. doi: 10.3201/eid1308.070315.

Baud, D. *et al.* (2014) 'Role of Waddlia chondrophila placental infection in miscarriage.', *Emerging infectious diseases*. Centers for Disease Control and Prevention, 20(3), pp. 460–4. doi: 10.3201/eid2003.131019.

Beatty, W. L., Byrne, G. I. and Morrison, R. P. (1994) 'Repeated and persistent infection with Chlamydia and the development of chronic inflammation and disease', *Trends in Microbiology*, 2(3), pp. 94–98. doi: 10.1016/0966-842X(94)90542-8.

Bertelli, C. *et al.* (2010) 'The Waddlia Genome: A Window into Chlamydial Biology', *PLoS ONE*. Edited by N. Ahmed. Horizon Bioscience, 5(5), p. e10890. doi: 10.1371/journal.pone.0010890.

Bolintineanu, D. S. and Kaznessis, Y. N. (2011) 'Computational studies of protegrin antimicrobial peptides: A review', *Peptides*, 32(1), pp. 188–201. doi: 10.1016/j.peptides.2010.10.006.

Braff, M. H. *et al.* (2005) 'Structure-Function Relationships among Human Cathelicidin Peptides: Dissociation of Antimicrobial Properties from Host Immunostimulatory Activities', *The Journal of Immunology*, 174(7).

Buchholz, K. R. and Stephens, R. S. (2006) 'Activation of the host cell proinflammatory interleukin-8 response by Chlamydia trachomatis', *Cellular Microbiology*. Blackwell Publishing Ltd, 8(11), pp. 1768–1779. doi: 10.1111/j.1462-5822.2006.00747.x.

Burton, M. J. *et al.* (2009) 'The Global Burden of Trachoma: A Review', *PLoS*

Neglected Tropical Diseases. Edited by S. Brooker. World Health Organization, 3(10), p. e460. doi: 10.1371/journal.pntd.0000460.

Ceovic, R. and Gulin, S. J. (2015) 'Lymphogranuloma venereum: diagnostic and treatment challenges.', *Infection and drug resistance*. Dove Press, 8, pp. 39–47. doi: 10.2147/IDR.S57540.

Chen, Y. *et al.* (2007) 'Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 51(4), pp. 1398–406. doi: 10.1128/AAC.00925-06.

Cheng, W. *et al.* (2008) 'Intracellular interleukin-1alpha mediates interleukin-8 production induced by Chlamydia trachomatis infection via a mechanism independent of type I interleukin-1 receptor.', *Infection and immunity*. American Society for Microbiology (ASM), 76(3), pp. 942–51. doi: 10.1128/IAI.01313-07.

Childs, T. *et al.* (2015) 'Rapid increase in lymphogranuloma venereum in men who have sex with men, United Kingdom, 2003 to September 2015', *Eurosurveillance*. European Centre for Disease Prevention and Control (ECDC), 20(48), p. 30076. doi: 10.2807/1560-7917.ES.2015.20.48.30076.

Chong-Cerrillo, C. *et al.* (2003) 'Susceptibility of human and murine Chlamydia trachomatis serovars to granulocyte- and epithelium-derived antimicrobial peptides', *The Journal of Peptide Research*. Munksgaard International Publishers, 61(5), pp. 237–242. doi: 10.1034/j.1399-3011.2003.00053.x.

Chopra, I. and Roberts, M. (2001) 'Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance.', *Microbiology and molecular biology reviews : MMBR*. American Society for Microbiology (ASM), 65(2), p. 232–60 ; second page, table of contents. doi: 10.1128/MMBR.65.2.232-260.2001.

Chromek, M. *et al.* (2006) 'The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection', *Nature Medicine*. Nature Publishing Group, 12(6), pp. 636–641. doi: 10.1038/nm1407.

Codony, F. *et al.* (2012) 'Well water as a possible source of Waddlia chondrophila infections.', *Microbes and environments*, 27(4), pp. 529–32.

Cole, J. N. and Nizet, V. (2016) 'Bacterial Evasion of Host Antimicrobial Peptide Defenses', in *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition*. American Society of Microbiology, pp. 413–443. doi: 10.1128/microbiolspec.VMBF-0006-2015.

Corsaro, D. and Greub, G. (2006) 'Pathogenic Potential of Novel Chlamydiae and Diagnostic Approaches to Infections Due to These Obligate Intracellular Bacteria', *Clinical Microbiology Reviews*, 19(2), pp. 283–297. doi: 10.1128/CMR.19.2.283-297.2006.

Coulon, C. *et al.* (2012) 'Amoebal host range, host-free survival and disinfection susceptibility of environmental *Chlamydiae* as compared to *Chlamydia trachomatis*', *FEMS Immunology & Medical Microbiology*, 64(3), pp. 364–373. doi: 10.1111/j.1574-695X.2011.00919.x.

Coyle, C. *et al.* (2016) 'Ovine trophoblasts express cathelicidin host defence peptide in response to infection', *Journal of Reproductive Immunology*, 117, pp. 10–16. doi: 10.1016/j.jri.2016.06.006.

Danka, E. S. and Hunstad, D. A. (2015) 'Cathelicidin augments epithelial receptivity and pathogenesis in experimental Escherichia coli cystitis.', *The Journal of infectious diseases*. Oxford University Press, 211(7), pp. 1164–73. doi: 10.1093/infdis/jiu577.

Darville, T. and Hiltke, T. J. (2010) 'Pathogenesis of genital tract disease due to Chlamydia trachomatis.', *The Journal of infectious diseases*. NIH Public Access, 201 Suppl 2(Suppl 2), pp. S114-25.

Dautry-Varsat, A., Subtil, A. and Hackstadt, T. (2005) 'Recent insights into the mechanisms of Chlamydia entry', *Cellular Microbiology*, 0(0), p. 051020044249005. doi: 10.1111/j.1462-5822.2005.00627.x.

Dawson, R. M. and Liu, C.-Q. (2008) 'Properties and Applications of Antimicrobial Peptides in Biodefense Against Biological Warfare Threat Agents', *Critical Reviews in Microbiology*, 34(2), pp. 89–107. doi: 10.1080/10408410802143808.

Dawson, R. M. and Liu, C.-Q. (2009) 'Cathelicidin peptide SMAP-29: comprehensive review of its properties and potential as a novel class of antibiotics', *Drug Development Research*. Wiley Subscription Services, Inc., A Wiley Company, 70(7), pp. 481–498. doi: 10.1002/ddr.20329.

Dilbeck, P. M. *et al.* (1990) 'Isolation of a previously undescribed rickettsia from an aborted bovine fetus.', *Journal of clinical microbiology*, 28(4), pp. 814–6.

Dille, S. *et al.* (2015) 'In contrast to Chlamydia trachomatis, Waddlia chondrophila grows in human cells without inhibiting apoptosis, fragmenting the Golgi apparatus, or diverting post-Golgi sphingomyelin transport.', *Infection and immunity*. American Society for Microbiology (ASM), 83(8), pp. 3268–80. doi: 10.1128/IAI.00322-15.

Donati, M. *et al.* (2005) 'Activity of Cathelicidin Peptides against Chlamydia spp.', *Antimicrobial Agents and Chemotherapy*, 49(3), pp. 1201–1202. doi: 10.1128/AAC.49.3.1201-1202.2005.

Donati, M. *et al.* (2007) 'Sensitivity of Chlamydia suis to cathelicidin peptides', *Veterinary Microbiology*, 123(1–3), pp. 269–273. doi: 10.1016/j.vetmic.2007.02.011.

Donati, M. *et al.* (2010) 'Increasing e¡ect of a high dose of PG-1peptide on the infectivity of Chlamydophila abortus'. doi: 10.1111/j.1574-695X.2010.00679.x.

Donati, M. *et al.* (2011) 'Activity of Cathelicidin Peptides against *Simkania negevensis*', *International Journal of Peptides*, 2011, pp. 1–3. doi: 10.1155/2011/708710.

Donati, M. *et al.* (2016) 'Tetracycline Susceptibility in Chlamydia suis Pig Isolates.', *PloS one*. Public Library of Science, 11(2), p. e0149914. doi: 10.1371/journal.pone.0149914.

Dürr, U. H. N., Sudheendra, U. S. and Ramamoorthy, A. (2006) 'LL-37, the only human member of the cathelicidin family of antimicrobial peptides', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1758(9), pp. 1408–1425. doi: 10.1016/j.bbamem.2006.03.030.

Elwell, C., Mirrashidi, K. and Engel, J. (2016) 'Chlamydia cell biology and pathogenesis', *Nature Reviews Microbiology*, 14(6), pp. 385–400. doi: 10.1038/nrmicro.2016.30.

Entrican, G. *et al.* (2004) 'Induction of inflammatory host immune responses by organisms belonging to the genera Chlamydia/Chlamydophila', *Veterinary Immunology*

and Immunopathology, 100(3–4), pp. 179–186. doi: 10.1016/j.vetimm.2004.04.006.

Everett, K. D. E. and Andersen, A. A. (1997) 'The Ribosomal Intergenic Spacer and Domain I of the 23s rRNA Gene Are Phylogenetic Markers for Chlamydia spp', *INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY*, 47(2), pp. 461–473.

Everett, K. D. E., Bush, R. M. and Andersen, A. A. (1999) 'Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms', *International Journal of Systematic Bacteriology*, 49(2), pp. 415–440. doi: 10.1099/00207713-49-2-415.

Fahrner, R. L. *et al.* (1996) 'Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes.', *Chemistry & biology*, 3(7), pp. 543–50.

Fehr, A. G. J. *et al.* (2016) 'A Zebrafish Model for Chlamydia Infection with the Obligate Intracellular Pathogen Waddlia chondrophila.', *Frontiers in microbiology*. Frontiers Media SA, 7, p. 1829. doi: 10.3389/fmicb.2016.01829.

Ganz, T. (2003) 'Defensins: antimicrobial peptides of innate immunity', *Nature Reviews Immunology*, 3(9), pp. 710–720. doi: 10.1038/nri1180.

Geisler, W. M. (2007) 'Management of Uncomplicated Chlamydia trachomatis Infections in Adolescents and Adults: Evidence Reviewed for the 2006 Centers for Disease Control and Prevention Sexually Transmitted Diseases Treatment Guidelines', *Clinical Infectious Diseases*, 44(Supplement 3), pp. S77–S83. doi: 10.1086/511421.

Geisler, W. M. *et al.* (2015) 'Azithromycin versus Doxycycline for Urogenital Chlamydia trachomatis Infection.', *The New England journal of medicine*. NIH Public Access, 373(26), pp. 2512–21. doi: 10.1056/NEJMoa1502599.

Gennaro, R., Skerlavaj, B. and Romeo, D. (1989) 'Purification, Composition, and Activity of Two Bactenecins, Antibacterial Peptides of Bovine Neutrophils', *INFECTION AND IMMUNITY*, 57(10), pp. 3142–3146.

Gervassi, A. *et al.* (2004) 'Differential regulation of inflammatory cytokine secretion by human dendritic cells upon Chlamydia trachomatis infection.', *Infection and immunity*. American Society for Microbiology (ASM), 72(12), pp. 7231–9. doi: 10.1128/IAI.72.12.7231-7239.2004.

Giakoumelou, S. *et al.* (2016) 'The role of infection in miscarriage.', *Human reproduction update*. Oxford University Press, 22(1), pp. 116–33. doi: 10.1093/humupd/dmv041.

Gordon, Y. J. *et al.* (2005) 'Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity.', *Current eye research*. NIH Public Access, 30(5), pp. 385–94. doi: 10.1080/02713680590934111.

Goy, G. and Greub, G. (2009) 'Antibiotic Susceptibility of Waddlia chondrophila in Acanthamoeba castellanii Amoebae', *Antimicrobial Agents and Chemotherapy*, 53(6), pp. 2663–2666. doi: 10.1128/AAC.00046-09.

Haggerty, C. L. *et al.* (2010) 'Risk of Sequelae after *Chlamydia trachomatis* Genital Infection in Women', *The Journal of Infectious Diseases*, 201(S2), pp. 134–155. doi: 10.1086/652395.

Haider, S. *et al.* (2008) 'Chlamydia-like bacteria in respiratory samples of community-acquired pneumonia patients', *FEMS Microbiology Letters*, 281(2), pp. 198–202. doi: 10.1111/j.1574-6968.2008.01099.x.

Hemshekhar, M., Anaparti, V. and Mookherjee, N. (2016) 'Functions of Cationic Host Defense Peptides in Immunity.', *Pharmaceuticals (Basel, Switzerland)*. Multidisciplinary Digital Publishing Institute (MDPI), 9(3). doi: 10.3390/ph9030040.

Heuer, D. *et al.* (2009) 'Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction', *Nature*. Nature Publishing Group, 457(7230), pp. 731–735. doi: 10.1038/nature07578.

HIRSCH, J. G. (1956) 'Phagocytin: a bactericidal substance from polymorphonuclear leucocytes.', *The Journal of experimental medicine*, 103(5), pp. 589–611.

Hogan, R. J. *et al.* (2004) 'Chlamydial persistence: beyond the biphasic paradigm.', *Infection and immunity*. American Society for Microbiology (ASM), 72(4), pp. 1843–55. doi: 10.1128/iai.72.4.1843-1855.2004.

Hvid, M. *et al.* (2007) 'Interleukin-1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection', *Cellular Microbiology*, 9(12), pp. 2795–2803. doi: 10.1111/j.1462-5822.2007.00996.x.

Hybiske, K. and Stephens, R. S. (2007) 'Mechanisms of host cell exit by the intracellular bacterium Chlamydia.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 104(27), pp. 11430–5. doi: 10.1073/pnas.0703218104.

Igietseme, J. U., Eko, F. O. and Black, C. M. (2011) '*Chlamydia* vaccines: recent developments and the role of adjuvants in future formulations', *Expert Review of Vaccines*. Taylor & Francis, 10(11), pp. 1585–1596. doi: 10.1586/erv.11.139.

Iimura, M. *et al.* (2005) 'Cathelicidin Mediates Innate Intestinal Defense against Colonization with Epithelial Adherent Bacterial Pathogens', *The Journal of Immunology*, 174(8).

Imtiaz, M. T. *et al.* (2006) 'Outcome of urogenital infection with Chlamydia muridarum in CD-14 gene knockout mice', *BMC Infectious Diseases*, 6(1), p. 144. doi: 10.1186/1471-2334-6-144.

Jacob, B. *et al.* (2014) 'Bacterial killing mechanism of sheep myeloid antimicrobial peptide-18 (SMAP-18) and its Trp-substituted analog with improved cell selectivity and reduced mammalian cell toxicity', *Amino Acids*, 46(1), pp. 187–198. doi: 10.1007/s00726-013-1616-8.

Jin, T. *et al.* (2004) 'Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism.', *Journal of immunology (Baltimore, Md. : 1950)*, 172(2), pp. 1169–76.

Kebbi-Beghdadi, C., Cisse, O. and Greub, G. (2011) 'Permissivity of Vero cells, human pneumocytes and human endometrial cells to Waddlia chondrophila', *Microbes and Infection*, 13(6), pp. 566–574. doi: 10.1016/j.micinf.2011.01.020.

Kokryakov, V. N. *et al.* (1993) 'Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins.', *FEBS letters*, 327(2), pp. 231–6.

Kong, F. Y. S. and Hocking, J. S. (2015) 'Treatment challenges for urogenital and

anorectal Chlamydia trachomatis.', *BMC infectious diseases*. BioMed Central, 15, p. 293. doi: 10.1186/s12879-015-1030-9.

Kościuczuk, E. M. *et al.* (2012) 'Cathelicidins: family of antimicrobial peptides. A review.', *Molecular biology reports*. Springer, 39(12), pp. 10957–70. doi: 10.1007/s11033-012-1997-x.

Kumar, Y. and Valdivia, R. H. (2008) 'Actin and intermediate filaments stabilize the Chlamydia trachomatis vacuole by forming dynamic structural scaffolds.', *Cell host & microbe*. NIH Public Access, 4(2), pp. 159–69. doi: 10.1016/j.chom.2008.05.018.

de la Maza, L. M., Zhong, G. and Brunham, R. C. (2017) 'Update on Chlamydia trachomatis Vaccinology', *Clinical and Vaccine Immunology*. Edited by C. J. Papasian, 24(4), pp. e00543-16. doi: 10.1128/CVI.00543-16.

Larrick, J. W. *et al.* (1996) 'Structural, functional analysis and localization of the human CAP18 gene.', *FEBS letters*, 398(1), pp. 74–80.

Li, H.-N. *et al.* (2009) 'Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages', *Journal of Leukocyte Biology*, 86(4), pp. 891–902. doi: 10.1189/jlb.0209050.

Lienard, J. *et al.* (2011) 'Development of a new chlamydiales-specific real-time PCR and its application to respiratory clinical samples.', *Journal of clinical microbiology*, 49(7), pp. 2637–42. doi: 10.1128/JCM.00114-11.

Malhotra, M. *et al.* (2013) 'Genital Chlamydia trachomatis: an update.', *The Indian journal of medical research*. Wolters Kluwer -- Medknow Publications, 138(3), pp. 303–16.

Massari, P. *et al.* (2013) 'Toll-like receptor 2-dependent activity of native major outer membrane protein proteosomes of Chlamydia trachomatis.', *Infection and immunity*. American Society for Microbiology (ASM), 81(1), pp. 303–10. doi: 10.1128/IAI.01062-12.

Matsumura, T. *et al.* (2016) 'Antimicrobial peptide LL-37 attenuates infection of hepatitis C virus', *Hepatology Research*, 46(9), pp. 924–932. doi: 10.1111/hepr.12627.

Mehlitz, A. and Rudel, T. (2013) 'Modulation of host signaling and cellular responses by Chlamydia.', *Cell communication and signaling : CCS*. BioMed Central, 11, p. 90. doi: 10.1186/1478-811X-11-90.

Menon, S. *et al.* (2015) 'Human and Pathogen Factors Associated with Chlamydia trachomatis-Related Infertility in Women.', *Clinical microbiology reviews*. American Society for Microbiology (ASM), 28(4), pp. 969–85. doi: 10.1128/CMR.00035-15.

Mookherjee, N. *et al.* (2006) 'Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37', *The Journal of Immunology*, 176(4).

Mookherjee, N. and Hancock, R. E. W. (2007) 'Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections', *Cellular and Molecular Life Sciences*, 64(7–8), pp. 922–933. doi: 10.1007/s00018-007-6475-6.

Moullan, N. *et al.* (2015) 'Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research.', *Cell reports*. NIH Public Access. doi: 10.1016/j.celrep.2015.02.034.

Mpiga, P. and Ravaoarinoro, M. (2006) 'Chlamydia trachomatis persistence: An

update', *Microbiological Research*, 161(1), pp. 9–19. doi: 10.1016/j.micres.2005.04.004.

Neth, O. W. *et al.* (2005) 'Susceptibility to infection in patients with neutropenia: the role of the innate immune system', *British Journal of Haematology*. Blackwell Science Ltd, 129(6), pp. 713–722. doi: 10.1111/j.1365-2141.2005.05462.x.

Niu, M. *et al.* (2015) 'Expression of porcine protegrin-1 in Pichia pastoris and its anticancer activity in vitro.', *Experimental and therapeutic medicine*. Spandidos Publications, 9(3), pp. 1075–1079. doi: 10.3892/etm.2015.2202.

O'Connell, C. M. *et al.* (2006) 'Localization of TLR2 and MyD88 to *Chlamydia trachomatis* Inclusions', *Journal of Biological Chemistry*, 281(3), pp. 1652–1659. doi: 10.1074/jbc.M510182200.

Paavonen, J. and Eggert-Kruse, W. (1999) 'Chlamydia trachomatis: impact on human reproduction', *Human Reproduction Update*, 5(5), pp. 433–447.

Parra-Sánchez, M. *et al.* (2016) 'Clinical and epidemiological characterisation of lymphogranuloma venereum in southwest Spain, 2013–2015', *Sexually Transmitted Infections*, 92(8), pp. 629–631. doi: 10.1136/sextrans-2015-052453.

Rasmussen, S. J. *et al.* (1997) 'Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 99(1), pp. 77–87. doi: 10.1172/JCI119136.

Resnikoff, S. *et al.* (2008) 'Global magnitude of visual impairment caused by uncorrected refractive errors in 2004.', *Bulletin of the World Health Organization*, 86(1), pp. 63–70.

Rurangirwa, F. R. *et al.* (1999) 'Analysis of the 16S rRNA gene of microorganism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddliaceae fam. nov., Waddlia chondrophila gen. nov., sp. nov.', *International Journal of Systematic Bacteriology*, 49(2), pp. 577–581. doi: 10.1099/00207713-49-2-577.

Saiman, L. *et al.* (2001) 'Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology, 45(10), pp. 2838–44. doi: 10.1128/AAC.45.10.2838-2844.2001.

Sandoz, K. M. and Rockey, D. D. (2010) 'Antibiotic resistance in Chlamydiae.', *Future microbiology*. NIH Public Access, 5(9), pp. 1427–42. doi: 10.2217/fmb.10.96.

Schachter, J., Causse, G. and Tarizzo, M. L. (1976) 'Chlamydiae as agents of sexually transmitted diseases.', *Bulletin of the World Health Organization*. World Health Organization, 54(3), pp. 245–54.

Schneider, V. A. F. *et al.* (2016) 'Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity', *Developmental & Comparative Immunology*, 60, pp. 108–114. doi: 10.1016/j.dci.2016.02.023.

Schoborg, R. V. (2011) 'Chlamydia persistence – a tool to dissect chlamydia–host interactions', *Microbes and Infection*, 13(7), pp. 649–662. doi: 10.1016/j.micinf.2011.03.004.

Scidmore, M. A. (2011) 'Recent advances in Chlamydia subversion of host cytoskeletal

and membrane trafficking pathways.', *Microbes and infection*. NIH Public Access, 13(6), pp. 527–35. doi: 10.1016/j.micinf.2011.02.001.

Scocchi, M. *et al.* (2005) 'Structural aspects and biological properties of the cathelicidin PMAP-36', *FEBS Journal*, 272(17), pp. 4398–4406. doi: 10.1111/j.1742-4658.2005.04852.x.

Scott, M. G. *et al.* (2000) 'Cutting Edge: Cationic Antimicrobial Peptides Block the Binding of Lipopolysaccharide (LPS) to LPS Binding Protein', *The Journal of Immunology*, 164(2).

Scott, M. G. *et al.* (2002) 'The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses', *The Journal of Immunology*, 169(7).

Shafer, W. M. (2006) *Antimicrobial peptides and human disease*. Springer.

Shinnar, A. E., Butler, K. L. and Park, H. J. (2003) 'Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance.', *Bioorganic chemistry*, 31(6), pp. 425–36.

Skerlavaj, B. *et al.* (1999) 'SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes', *FEBS Letters*, 463(1–2), pp. 58–62. doi: 10.1016/S0014-5793(99)01600-2.

Soper, D. E. (2010) 'Pelvic Inflammatory Disease', *Obstetrics & Gynecology*, 116(2, Part 1), pp. 419–428. doi: 10.1097/AOG.0b013e3181e92c54.

Sørensen, O. *et al.* (1997) 'The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils.', *Blood*, 90(7), pp. 2796–803.

Sorensen, O. and Borregaard, N. (2005) 'Cathelicidins - Natures Attempt at Combinatorial Chemistry', *Combinatorial Chemistry & High Throughput Screening*, 8(3), pp. 273–280. doi: 10.2174/1386207053764602.

Sørensen, O. E. *et al.* (2001) 'Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3.', *Blood*, 97(12), pp. 3951–9.

Sousa, F. H. *et al.* (2017) 'Cathelicidins display conserved direct antiviral activity towards rhinovirus.', *Peptides*, 95, pp. 76–83. doi: 10.1016/j.peptides.2017.07.013.

Steinstraesser, L. *et al.* (2001) 'Protegrin-1 enhances bacterial killing in thermally injured skin.', *Critical care medicine*, 29(7), pp. 1431–7.

Stephens, R. *et al.* (1999) 'Comparative genomes of Chlamydia pneumoniae and C. trachomatis.', *Nature Genetics*, 21(4), pp. 385–389. doi: 10.1038/7716.

Storrie, S. *et al.* (2016) 'MAPK Activation Is Essential for Waddlia chondrophila Induced CXCL8 Expression in Human Epithelial Cells', *PLOS ONE*. Edited by D. M. Ojcius, 11(3), p. e0152193. doi: 10.1371/journal.pone.0152193.

Suchland, R. J. *et al.* (2009) 'Horizontal transfer of tetracycline resistance among Chlamydia spp. in vitro.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology, 53(11), pp. 4604–11. doi: 10.1128/AAC.00477-09.

Tack, B. F. *et al.* (2002) 'SMAP-29 has two LPS-binding sites and a central hinge.', *European journal of biochemistry*, 269(4), pp. 1181–9.

Tang, X. *et al.* (2015) 'P2X7 Receptor Regulates Internalization of Antimicrobial Peptide LL-37 by Human Macrophages That Promotes Intracellular Pathogen Clearance.', *Journal of immunology (Baltimore, Md. : 1950)*. The American Association of Immunologists, Inc., 195(3), pp. 1191–201. doi: 10.4049/jimmunol.1402845.

Thomassin, J.-L. *et al.* (2012) 'OmpT Outer Membrane Proteases of Enterohemorrhagic and Enteropathogenic Escherichia coli Contribute Differently to the Degradation of Human LL-37', *Infection and Immunity*, 80(2), pp. 483–492. doi: 10.1128/IAI.05674-11.

Travassos, L. H. *et al.* (2010) 'Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry', *Nature Immunology*, 11(1), pp. 55–62. doi: 10.1038/ni.1823.

Travis, S. M. *et al.* (2000) 'Bactericidal activity of mammalian cathelicidin-derived peptides.', *Infection and immunity*. American Society for Microbiology (ASM), 68(5), pp. 2748–55.

Tripathi, S. *et al.* (2014) 'LL-37 modulates human neutrophil responses to influenza A virus.', *Journal of leukocyte biology*. The Society for Leukocyte Biology, 96(5), pp. 931–8. doi: 10.1189/jlb.4A1113-604RR.

Turner, J. *et al.* (1998) 'Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils.', *Antimicrobial agents and chemotherapy*, 42(9), pp. 2206–14.

Vandamme, D. *et al.* (2012) 'A comprehensive summary of LL-37, the factotum human cathelicidin peptide', *Cellular Immunology*, 280(1), pp. 22–35. doi: 10.1016/j.cellimm.2012.11.009.

Vasilevsky, S. *et al.* (2015) 'Waddlia chondrophila induces systemic infection, organ pathology, and elicits Th1-associated humoral immunity in a murine model of genital infection.', *Frontiers in cellular and infection microbiology*. Frontiers Media SA, 5, p. 76. doi: 10.3389/fcimb.2015.00076.

Verweij, S. P. *et al.* (2015) 'Waddlia chondrophila and Chlamydia trachomatis antibodies in screening infertile women for tubal pathology', *Microbes and Infection*, 17(11–12), pp. 745–748. doi: 10.1016/j.micinf.2015.09.019.

Wang, G. (2008) 'Structures of Human Host Defense Cathelicidin LL-37 and Its Smallest Antimicrobial Peptide KR-12 in Lipid Micelles', *Journal of Biological Chemistry*, 283(47), pp. 32637–32643. doi: 10.1074/jbc.M805533200.

Wang, Y. *et al.* (2017) 'Chlamydial Lipoproteins Stimulate Toll-Like Receptors 1/2 Mediated Inflammatory Responses through MyD88-Dependent Pathway', *Frontiers in Microbiology*. Frontiers, 8, p. 78. doi: 10.3389/fmicb.2017.00078.

Wheelhouse, N. *et al.* (2009) 'Ovine trophoblast is a primary source of TNFα during Chlamydophila abortus infection', *Journal of Reproductive Immunology*, 80(1–2), pp. 49–56. doi: 10.1016/j.jri.2008.12.003.

Wheelhouse, N. *et al.* (2014) 'Waddlia chondrophila Infects and Multiplies in Ovine Trophoblast Cells Stimulating an Inflammatory Immune Response', *PLoS ONE*. Edited by B. Kaltenboeck, 9(7), p. e102386. doi: 10.1371/journal.pone.0102386.

Workowski, K. A., Bolan, G. A. and Centers for Disease Control and Prevention (2015) 'Sexually transmitted diseases treatment guidelines, 2015.', *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports*,

64(RR-03), pp. 1–137.

Wyrick, P. B. (2010) ‘Chlamydia trachomatis persistence in vitro: an overview.’, *The Journal of infectious diseases*. NIH Public Access, 201 Suppl 2(Suppl 2), pp. S88-95. doi: 10.1086/652394.

Yang, D. *et al.* (2004) ‘Multiple Roles of Antimicrobial Defensins, Cathelicidins, and Eosinophil-Derived Neurotoxin in Host Defense’, *Annual Review of Immunology*, 22(1), pp. 181–215. doi: 10.1146/annurev.immunol.22.012703.104603.

Yang, D., Chertov, O. and Oppenheim, J. J. (2001) ‘Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37).’, *Journal of leukocyte biology*, 69(5), pp. 691–7.

Yasin, B. *et al.* (1996) ‘Susceptibility of Chlamydia trachomatis to protegrins and defensins.’, *Infection and immunity*, 64(3), pp. 709–13.

Zanetti, M. *et al.* (1994) ‘Molecular cloning and chemical synthesis of a novel antibacterial peptide derived from pig myeloid cells.’, *The Journal of biological chemistry*, 269(11), pp. 7855–8.

Zheng, Y. *et al.* (2007) ‘Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human α-defensins from neutrophils’, *British Journal of Dermatology*, 157(6), pp. 1124–1131. doi: 10.1111/j.1365-2133.2007.08196.x.

Zhong, G. (2009) ‘Killing me softly: chlamydial use of proteolysis for evading host defenses.’, *Trends in microbiology*. NIH Public Access, 17(10), pp. 467–74. doi: 10.1016/j.tim.2009.07.007.

Ziklo, N. *et al.* (2016) ‘Chlamydia trachomatis Genital Tract Infections: When Host Immune Response and the Microbiome Collide’, *Trends in Microbiology*, 24(9), pp. 750–765. doi: 10.1016/j.tim.2016.05.007.

Zou, Y. *et al.* (2016) ‘The role of NOD1 and NOD2 in host defense against chlamydial infection’, *FEMS Microbiology Letters*, 363. doi: 10.1093/femsle/fnw170.

Zughaier, S. M. *et al.* (2010) ‘The Human Host Defense Peptide LL-37 Interacts with Neisseria meningitidis Capsular Polysaccharides and Inhibits Inflammatory Mediators Release’, *PLoS ONE*. Edited by D. Unutmaz, 5(10), p. e13627. doi: 10.1371/journal.pone.0013627.

Zuyderduyn, S. *et al.* (2006) ‘The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells.’, *The Journal of allergy and clinical immunology*, 117(6), pp. 1328–35. doi: 10.1016/j.jaci.2006.03.022.