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Targeted amplification of the oxidative burst in neutrophils as a bactericidal strategy against uropathogenic *Escherichia coli*

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Abstract:

Urinary tract infections (UTIs), commonly caused by the Gram-negative bacteria *Escherichia. coli,* affect 50% of women and 12% of men in their lifetimes and represent an enormous burden on healthcare providers. UTIs have a high rate of recurrence and a low rate of resolution without antibiotic intervention, making them a driving force in the rise of antimicrobial resistance (AMR). Alternative therapies such as immunomodulation could aid in reduction of antibiotic prescription and AMR. Small molecule compounds RE-04-001 and RE-04-006 selectively target Formyl peptide receptor 1 (FPR1) in neutrophils, the most abundant innate immune cells in UTIs, resulting in the generation of toxic reactive oxygen species via the oxidative burst. This research aimed to characterize interactions between FPR1-stimulated neutrophils and Uropathogenic *E. coli* (UPEC). Neutrophils isolated from whole blood were co-cultured with UPEC strain CFT-073 or laboratory strain K12 and co-treated with FPR1-stimulating RE-04-001, RE-04-006 or N-Formylmethionyl-leucyl-phenylalanine. The internal killing ability of neutrophils was assessed using a gentamicin protection assay. Light microscopy was used to investigate phagocytosis. Neutrophil morphology following FPR1 stimulation was observed using flow cytometry, and viability was tested with DRAQ7™. Finally, confocal microscopy assessed FPR1-driven NETosis. FPR1 stimulation was found to enhance intracellular killing of CFT-073 but not K12. Extracellular killing of K12 but not UPEC CFT-073 in the supernatant was enhanced by FPR1 activation. FPR1 stimulation was found to have no significant effects on the phagocytosis of *E. coli*, contrasting other findings in the literature. FPR1-stimulating compounds had no impact on neutrophil viability but caused a significant shift in their morphology. Finally, FPR1 stimulation was observed to induce NETosis in neutrophils after 3 hours but no NETosis was recorded in co-cultures with *E. coli*. This work highlights FPR1 as a target for immunostimulatory therapy which is a critical alternative to or co-therapy with antibiotic treatment in UTIs.

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

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Abbreviations

8-oxo-G	8-oxo-7,8-dihydroguanosine
Ahp	Alkyl hydroperoxide reductase
AMPK	AMP Kinase
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
BEC	Bladder epithelial cell
BSA	Bovine serum albumin
CFU	Colony forming units
CGD	Chronic granulomatous disease
CR	Complement receptor
DAG	Diacyl glycerol
DAHP	3-Deoxy-D-arabinoheptulosonate 7-phosphate
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EF	Elongation factor
ExPEC	Extraintestinal pathogenic *E. coli*
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FcγR	Fcγ receptors
fMLF	N-Formylmethionyl-leucyl-phenylalanine
FPR1	Formyl peptide receptor 1
FSC	Forward scatter
GDI	GDP dissociation inhibitor
GEF	Guanine exchange factor
GPCR	G-protein coupled receptor
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HOCl	Hypochlorous acid

HRP	Peroxidase type II from horseradish
IBC	Intracellular bacterial community
IL	Interleukin
IP_3	Inositol triphosphate
IPEC	Intestinal pathogenic *E. coli*
LB	Lysogeny broth
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MID	Microbiology and infectious disease
MMP9	Metallomatrix proteinase 9
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NET	Neutrophil extracellular trap
NHS	National health service
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer cells
NOX2	NADPH oxidase 2
PAMP	Pathogenic associated molecular pattern
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PREX1	PIP_3 dependent Rac exchange factor 1
PRR	Pattern recognition receptor
QIR	Quiescent intracellular reservoir
Rac	Ras-related C3 botulinum toxin substrate
ROS	Reactive oxygen species

S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. lugdunensis	*Staphylococcus lugdunensis*
SEM	Standard error of the mean
SOD	Superoxide dismutase
SSC	Side scatter
SV	Secretory vesicle
TLR	Toll like receptor
TNF- α	Tumour necrosis factor alpha
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VF	Virulence factor

Chapter 1: Introduction

1.1 Urinary Tract Infections

Urinary tract infections (UTIs) represent a significant burden on the healthcare system in the United Kingdom. From 2018 to 2023, UTIs were responsible for almost 2 million hospital admissions in the UK alone, costing the National Health Service (NHS) £500 million annually, a figure not including UTIs resolved by GPs or pharmacists (Brine, 2018; Wilkinson, 2023). A urinary tract infection is an infection of the urethra, bladder, ureter, or kidneys, affecting up to 50% of women and 12% of men within their lifetimes. Of those affected, 30% will additionally suffer from a recurrent infection, defined as a reinfection 6 months after the initial infection or the initial infection becoming chronic (Foxman and Chi, 1990). UTI prevalence increases with age, being twice as frequent in women over 65 than the rest of the female population (Rowe and Juthani-Mehta, 2013). Infections of the lower urinary tract such as the urethra (urethritis) and bladder (cystitis) can be uncomfortable and disruptive to everyday life but can typically be resolved by the immune system and antibiotics (Bono *et al.*, 2024). If left untreated, the infection can ascend the urinary tract where it can become a more significant problem. Higher UTIs involving the ureters or kidneys (pyelonephritis) can lead to permanent kidney damage, renal abscesses, and, if the infection is able to enter the blood (bacteraemia), it may cause sepsis (Dagasso *et al.*, 2018). Indeed, 30% of all sepsis cases originate in the urinary tract (Wagenlehner *et al.*, 2015). Other co-morbidities can have a notable impact on the effectiveness of UTI treatment and the severity of the disease. So called complicated UTIs occur in patients possessing another condition which may impede the treatment or exacerbate the symptoms of a UTI, such as diabetes, chronic renal cancer of the urinary tract, anatomical abnormalities such as a fistula and pregnancies (Wagenlehner *et al.*, 2020).

1.2 *Escherichia coli*

1.2.1 The Causes of UTIs

Bacteria are the major causative organism of UTIs, being responsible for 90-95% of all cases. The remaining cases are caused by fungi such as *Candida albicans* or more rarely viruses (Flores-Mireles *et al.*, 2015). The bacteria *Escherichia coli* is estimated to cause 80-90% of UTIs (Foxman and Brown, 2003). *E. coli* is a Gram-negative, rod-shaped, facultative anaerobe and a typical member of the intestinal microbiota, where it is thought to colonize within a few hours of birth (Mackie *et al.*, 1999). Non-pathogenic *E. coli* thrive within their

niche of the mucous layer of the colon, forming the majority of facultative anaerobes within this region and representing 0.1% of the total gut flora (Dubreuil, 2014). Non-pathogenic *E. coli* is unable to breach the intestinal epithelium or form viable colonies outside of its niche and can therefore do little to harm its host (Martinson and Walk, 2020). Some strains can even provide benefits to the host by providing colonisation resistance (Maltby *et al.*, 2013). However, certain strains of *E. coli* have acquired specific cellular structures, systems, or molecules, known as virulence factors (VFs), which allow them to exist and even thrive outside of their intestinal niche (Kaper, Nataro and Mobley, 2004). These pathogenic strains, or pathovars, can thereby escape the mucosal niche, evade the immune system, and colonise different regions of the body, ultimately causing disease.

Infectious *E. coli* pathovars can be broadly classified into two groups: intestinal (or diarrheagenic) and extraintestinal. Intestinal pathogenic *E. coli* (IPEC) predominantly infect the gastrointestinal tract and are grouped for their propensity to cause loose bowel movements amongst other gastrointestinal symptoms. These pathovars have acquired VFs that allow them to better adhere to and sometimes breach the intestinal wall. IPEC is responsible for many foodborne illnesses and conditions such as traveller's diarrhoea (Nataro and Kaper, 1998).

Extraintestinal pathogenic *E. coli* (ExPEC) are responsible for infections outside of the intestine including the brain, the lungs, the blood and importantly the urogenital tract (Russo and Johnson, 2000). Uropathogenic *E. coli* (UPEC) is an ExPEC pathovar capable of infecting the urinary tract, bladder, prostate, and kidneys (Kaper, Nataro and Mobley, 2004). UPEC can be found within the intestine and faeces but must find its way to the urinary tract in order to trigger an infection. This transfer can occur during defecation, sexual intercourse or washing with contaminated water (Kaper, Nataro and Mobley, 2004). One reason as to why women are more susceptible to UTIs is that their anatomy makes this transfer much easier; women have both a shorter urethra than men and a shorter distance between the anus and the urethra allowing faecal bacteria more opportunities to access the urinary tract (Minardi *et al.*, 2011).

1.2.2 UPEC Pathogenesis

UPEC face a far more daunting challenge than their diarrheagenic 'cousins'; rather than merely exploiting the intestinal niche in a more aggressive fashion, UPEC must escape the intestine, make their way to the urethra, and ascend into the bladder and kidneys, whilst evading urogenital immunity. To achieve this, UPEC strains possess many important VFs

which have allowed them to adapt to survival and invasion of the urogenital tract (Table 1.1; Figure 1.1).

Table 1.1. Virulence factors associated with UPEC

Name	Genes	Function	Reference
Adhesion and movement			
Type 1 fimbria	*fimA-H*	Tipped with FimH which blinds to bladder surface proteins such as Uroplakins a3 and b1, enabling cell infiltration	Zhou *et al.*, 2001
Curli	*csgA-G*	Enhances biofilm formation	Goyal *et al.*, 2014
P fimbria	*papA-J*	Tipped with PapG which binds to globoside molecules on kidney cells, allowing passage into the collecting ducts	Lane and Mobley, 2007
dr fimbriae	*draA-E*	Tipped with DraE which binds to kidney immune proteins, enabling invasion	Goluszko *et al.*, 1997
Hemagglutin	*ha*	Enhances adhesion and biofilm formation	Maheswari *et al.*, 2013
Flagella	*flhA-E*	Whip-like appendages which propel bacteria through the urinary tract	Wright, Seed and Hultgren, 2005
Toxins			
Haemolysin A	*hlyA*	Disrupts host cell membranes triggering cell death	Dhakal and Mulvey, 2012
Cytolytic Necrotizing Factor 1	*cnf1*	Induces membrane ruffling and inhibits BEC cell death, enhancing *E. coli* invasion	Davis, Rasmussen and O'Brien, 2005
Serine protease autotransporter	*vat, pic*	Cleave host immune proteins and surface proteins enhancing invasion	Parham *et al.*, 2005
Cytolethal distending toxin	*cdtA-C*	Arrest the cell cycle in host cells, triggering apoptosis	Tóth *et al.*, 2003
Secreted Autotransporter Toxin	*sat*	Breaks down complement proteins and cytokines	Guyer *et al.*, 2002
Siderophores			
Iron response element A	*ireA*	Scavenges ferric iron from the extracellular space	Hagan and Mobley, 2007
Hemin receptor	*chuA*	Scavenges iron from host haemoglobin	Spurbeck *et al.*, 2012
aerobactin	*iucA-D, iutA*	Scavenges ferric iron from the extracellular space and transports it into the cytoplasm	Johnson *et al.*, 1988
enterobactin	*entA-F*	Sequesters iron from the extracellular space	Raymond, Dertz and Kim, 2003
Protectins			
Capsule	*kps*	Crucial in the formation of Intercellular Bacterial Communities	Anderson *et al.*, 2010
Serum resistance protein	*traT*	Cleaves complement proteins, inhibiting phagocytosis	Derakhshandeh *et al.*, 2015
Outer membrane protein	*ompA*	Cleaves AMPs, enhancing survival	Rodrigues *et al.*, 2022

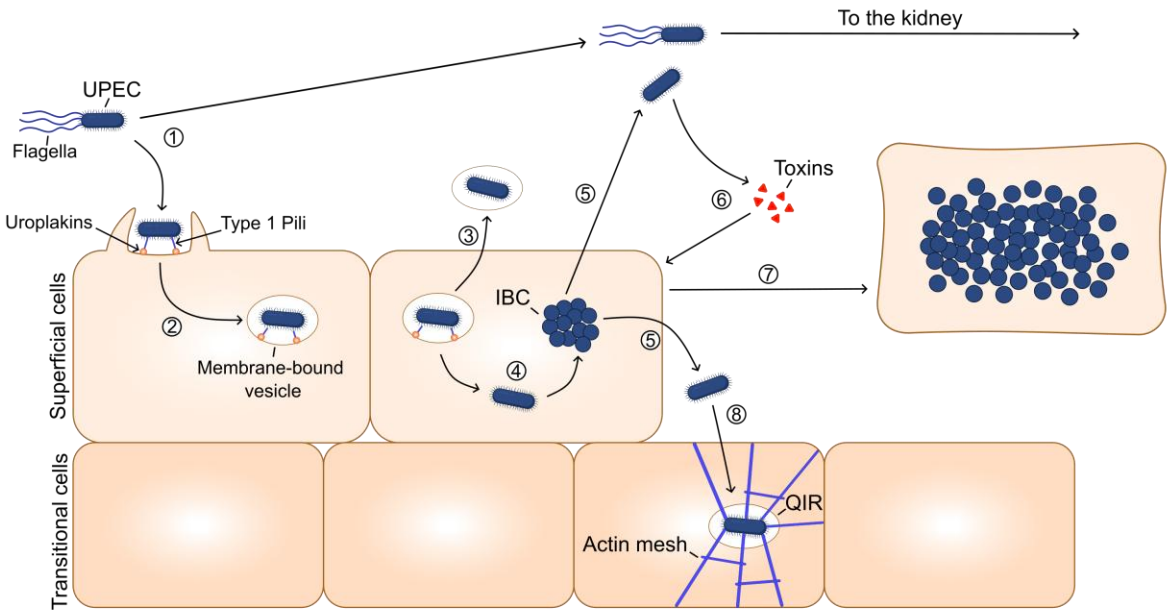

Figure 1.1 Mechanisms of UPEC infection in the bladder

1. UPEC ascends the urinary tract into the bladder using their flagella. Once inside UPEC can adhere to uroplakin proteins on the bladder epithelium with fimbriae or continue their ascent towards the kidney. 2. Adhesion triggers internalisation of UPEC via a membrane-bound vesicle. 3. Upon detecting UPEC, the bladder cell will traffic the vesicle back to the surface, expelling the bacteria 4. UPEC escapes the vesicle before expulsion. UPEC can then rapidly replicate into an IBC within the cytoplasm. 5. The IBC serves as a reservoir of infection allowing UPEC to efflux back into the lumen or into surrounding cells. 6. The growing bacterial population releases toxins into their surroundings. 7. Damage caused by toxins and inflammation triggers the exfoliation of bacteria-laden superficial BECs, decreasing the bacterial load of the bladder but exposing the vulnerable transitional epithelium. 8. UPEC invades the transitional epithelium, the invading vesicle is enmeshed in actin forming a quiescent intracellular reservoir (QIR), which can avoid the immune system long after the initial infection. Adapted from Flores-Mireles *et al.*, 2015

Upon entering the urinary tract, UPEC will ascend the urethra using their flagella, whip-like appendages which protrude from its surface and propel the bacteria (Lane *et al.*, 2007). To prevent itself from being swept out of the bladder by urine flowing in the opposite direction, UPEC adheres to the urothelium with specialised appendages called fimbria. These fimbriae are tipped with adhesive proteins which can bind to specific host motifs (Klemm, 1985). The host receptor profile of the urinary tract changes as the bacterium ascends, so different types of fimbriae are employed in the bladder, ureters and kidneys (Lane and Mobley, 2007). Adhesion also allows *E. coli* to invade bladder epithelial cells (BECs). The mechanisms behind this invasion are not fully understood, however it is believed that UPEC hijacks the

natural trafficking of specialised bladder proteins called uroplakins to enter via a vesicle (Thumbikat *et al.*, 2009). UPEC can then breach this vesicle and enter the BEC cytoplasm where they will rapidly replicate and undergo a series of morphological changes, forming a biofilm-like population known as an intracellular bacterial community (IBC; Justice *et al*., 2004). These are an important reservoir for UPEC and provide them with a protected niche within which they can proliferate and disseminate into surrounding cells. To prevent the formation of IBCs, under prolonged stress signalling the urothelium will undergo a form of controlled inflammatory cell death called pyroptosis causing the superficial layer of the BECs to shed from of the bladder, significantly reducing the bacterial load (Jones *et al.*, 1997). Some strains of UPEC will secrete toxins into the bladder environment which act to damage BECs and immune cells or enhance BEC invasion, these can also contribute to shedding (Mysorekar and Hultgreen, 2006). While shedding can initially prove to be a large setback to UPEC invasion, it also exposes the more vulnerable underlying immature transitional epithelial cells. These developing cells have a dense actin cytoskeleton causing invading bacteria to become enmeshed in actin within the vesicle (Eto, Sundsbak and Mulvey, 2006). This forms what is known as a QIR, wherein a small community of 5-10 bacteria enter a dormant state, replicating extremely slowly and lowering their metabolic activity (Mysorekar and Hultgreen, 2006). Within a QIR, bacteria can avoid immune detection and are more resistant to antibiotics. Bacteria can persist in QIRs and escape into the surrounding tissue long after the initial infection (Blango *et al.*, 2014).

The ability of UPEC to invade bladder cells and avoid the immune system likely contribute to the frequency of recurrent and chronic UTIs. The formation of IBCs is a rare event; murine experiments involving inoculation with 10^7 bacteria observed only 500 IBC, however, a single IBC can contain up to 10^5 bacteria serving as a formidable reservoir for perpetuating or reinitiating infections (Justice *et al.*, 2004). The longer lived QIRs make up only 15% of intracellular UPEC, however their considerable longevity and resistance to treatment allow them to lie undetected for weeks to months, wherein they can reemerge within the urinary tissue to begin a new cycle of infection (Kim *et al.*, 2021).

Once UPEC has established a foothold in the bladder it will ascend the ureters into the kidneys. Again, UPEC will adhere to kidney cells to prevent expulsion; however, unlike within the bladder, UPEC do not seem to form IBCs within kidney cells instead focusing on translocation through the epithelial cells of collecting ducts, into the renal interstitium and

then ultimately into the bloodstream (Chassin *et al.*, 2008). Bacteraemia can have serious health consequences, including sepsis which can be fatal if not treated quickly.

1.2.3 Antimicrobial Resistance

Antimicrobial resistance (AMR) is an increasingly prevalent and pertinent issue associated with UTIs. AMR-associated infections contribute to 1.5 million deaths annually and have done every year since 1990. They are additionally implicated in another 5 million deaths a year as complicating agents, increasing to 8 million by 2050 (Naghavi *et al*., 2024). In the UK, *E. coli* is the dominant source of antibiotic-resistant bacteraemia, making up 83.4% of all cases in 2023, a 10% increase from 2019 (UKHSA, 2023). The World Health Organisation found that globally 20% of UTIs are resistant to commonly prescribed antibiotics such as ampicillin, and that 90% of *E. coli* infections are resistant to at least one antibiotic (WHO, 2022). UPEC is known to possess a large array of beta-lactamases which interfere with the most common types of antibiotics. Similarly, mutations in Topoisomerase IV and DNA gyrase grant UPEC considerable resistance to quinolones and fluoroquinolones (Sora *et al*., 2021; Carter *et al*., 2023). AMR is exacerbated by over-prescription of antibiotics and improper use (Llor and Bjerrum, 2014).

1.3 The Innate Immune System

The innate immune system represents the first line of defence encountered by pathogens. Innate immunity is formed of two major arms; the innate barriers such as mucus membranes which physically or chemically prevent pathogens from entering the body, and the cellular arm composed of resident and patrolling non-specific immune cells which will initiate inflammation, destroy pathogens and activate specific adaptive immunity if necessary. The innate immune system is also crucial in repairing damage caused by pathogens during and after infection, influencing differentiation of new healthy cells and angiogenesis to restore damaged tissue.

1.3.1 Barrier Immunity

The first obstacle encountered by UPEC are barriers which serve to physically inhibit bacterial entry to the host whilst also reducing bacterial viability and survival. The urothelium represents the first barrier in the urinary tract, a stratified epithelial cell layer joined by tight junctions which lines the inner surface of the bladder which bacteria cannot cross freely. The urothelium is a robust, waterproof barrier that can withstand the harsh environment of the

bladder, including strong stretch forces, large changes in osmotic pressure and toxic components of the urine (Jafari and Rohn, 2022). The bladder is additionally lined with a layer of thick mucus which can trap bacteria allowing it to be swept away by urine (Fry and Bahabi, 2016). Urine itself also has mild antibacterial properties, possessing a low pH and a high concentration of toxic urea which can inhibit bacterial growth (Ipe, Horton and Ulett, 2016). Urine, and by extension the bladder, is often mischaracterized as a sterile environment. The bladder supports its own, if modest, microbiota which like all microbiotas will impede attempts at colonization by other bacteria, including UPEC (Perez-Carrasco *et al.*, 2021). Indeed, the bladder microbiota can have mild bactericidal effects. *Lactobacillus*, found commonly in women's bladders releases lactic acid, lowering the pH of urine and thereby impeding bacterial growth (Vagios, Hesham and Mitchell, 2020). To contain this microbiota at tolerable levels and to prevent the invasion of pathogenic bacteria, urogenital cells constitutively release a low level of antimicrobial peptides (AMPs) into the bladder and kidneys, inhibiting bacterial function and growth. Tamm-Horsfall protein, or uromodulin, is produced by epithelial cells in the kidney and diffuses throughout the urinary tract. Uromodulin acts on bacteria by strongly binding to type 1 fimbriae, preventing them from interacting with host cells and halting bacterial invasion. These unbound bacteria can then be swept out of the bladder by micturition (Pennica *et al.*, 1987). Cathelicidin has a more direct impact on bacteria by forming pores in the bacterial membrane, disrupting their stability and potentially killing the cell (Harten *et al.*, 2018). Cathelicidin along with many other AMPs also have immunomodulatory effects, aiding in tissue repair, innate cell recruitment and inflammation (Guryanova and Ovchinnikova, 2022)

1.3.2 Toll-like Receptors

The urinary tract has significant levels of 'sensing' receptors which detect 'danger'. Microbes will activate these receptors which will trigger the beginning of an immune response. The Toll-like receptors (TLRs) are a vital component of the innate immune response which line the surface of the urothelium as well as various immune cells such as neutrophils (Medzhitov, 2001). Toll-like receptors are activated by specific pathogenic motifs, termed pathogen associated molecular patterns (PAMPs), allowing them to orchestrate an appropriate immune reaction depending on the nature of the pathogen. For example, TLR4 recognises lipopolysaccharide, a component of Gram-negative bacterial cell walls and thus relevant to UPEC (Park and Lee, 2013). TLR4 can be found on the surface of immune and bladder cells and within bacteria-containing vesicles. Its activation enhances the expulsion of these

vesicles into the extracellular space, decreasing the bacterial load of the cell. This is an extremely sensitive mechanism; studies have demonstrated that UPEC expulsion can be detected within minutes of infection, and throughout the course of the infection almost 70% of all bacteria is expelled back into the bladder (Bishop *et al.*, 2007).

1.3.3 Cytokines

Cytokines are small immunomodulatory proteins that facilitate communication between non-immune and immune cells as well as intercommunication within the innate and adaptive immune system. Cytokines are responsible for the activation, persistence and clearance of immune cells during an inflammatory response. Chemokines, a subset of cytokines are crucial to the recruitment of immune cells to the site of infection (Zhang and An, 2007). Different cytokines are expressed and accumulate at different stages of a UTI dictating a pro- or anti-inflammatory response. Activation of TLR4 with downstream activation of the transcription factor NF-κB promotes early expression of cytokines in the form of interleukin (IL) 6, IL-18, IL-1β and tumour necrosis factor α (TNF-α) roughly 1 hour after TLR-4 activation (Mizgerd *et al.*, 2004). These function as pro-inflammatory mediators which act to recruit and activate effector cells like neutrophils and induce other immune functions such as the complement system or acute phase response (Gruys *et al.*, 2005).

1.3.4 Resident Immune Cells

Throughout the layers of the urothelium are a host of resident immune cells such as macrophages, mast cells and natural killer (NK) cells which are constantly monitoring the local environment for infection and poised for a rapid response should they encounter a pathogen. Like the cells of the urothelium, they are lined with TLRs alongside other pattern recognition receptors (PRRs) and will release large quantities of pro-inflammatory cytokines in the event of an infection, as well as aiding in the recruitment and activation of innate effector cells such as neutrophils and the destruction of bacteria (Ingersol and Albert, 2013).

1.4 Neutrophils

1.4.1 Neutrophil Recruitment

Neutrophils are the first effector cells to arrive at the bladder during a UTI, detectable in the urine after only 2 hours; within the bladder they act as the primary effector cell throughout the infection acting to engulf and destroy bacteria (Agace *et al.*, 1995). Beyond the simple destruction of bacteria, neutrophils are also becoming increasingly recognized as key effector

cells in the resolution of inflammation, and important immunomodulators helping to sculpt innate immunity towards the most effective response (Lee, Lee and Bae, 2022). Neutrophils are phagocytic leukocytes and one of the predominant cells within the innate immune system, making up 40-70% of the blood leukocyte population (Borregaard, 2010). Like all leukocytes, neutrophils are formed in the bone marrow and patrol the bloodstream in an inactivated state until recruited to the site of infection. While in this state neutrophils are extremely short lived, with lifespans as low as 6 hours (Tak *et al.*, 2013). Neutrophils are recruited from the blood by the actions of chemokines such as IL-8 which upregulate the expression of adhesion molecules such as E-selectin on the vascular endothelium that can bind neutrophils in the blood, allowing them to transmigrate the endothelium into the tissue. They migrate along an increasing gradient of chemokines towards the site of infection such as the bladder (Haraldsen *et al*., 1996; Hayashi *et al*., 2003).

1.4.2 Neutrophil Activation

Neutrophil activation is a multi-step process. A single stimulus is not sufficient to activate a neutrophil but instead leaves it in a primed state where it is ready to mount a rapid inflammatory response upon encountering a second stimulus. This 'double strike' system is important in preventing an inappropriate inflammatory response in healthy regions of the body. During a UTI, neutrophils will typically encounter their first signal in the form of a chemokine such as IL-8. These are generated by the local bladder immune system and acts to prime and recruit the neutrophil. The second signal will be encountered much closer to the site of infection bladder as either a pathogenic motif such as lipopolysaccharide (LPS), or another immune signal such as a cytokine. Pathogenic motifs are recognised by PRRs such as the TLRs, or importantly by G protein-coupled receptors (GPCRs) such as formyl peptide receptor 1 (FPR1). Immune motifs such as complement proteins or antibodies are recognised by complement receptors (CR) and Fcγ receptors (FcγR) respectively (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002).

1.4.3 Neutrophil Immunomodulation

Neutrophils have been observed to produce an array of different cytokines following activation (Sollberger *et al*., 2024). The cytokine profile of neutrophils will vary depending on the state and timeframe of infection; for instance early in the infection neutrophils will secrete chemokines like IL-8 and CXCL11 to recruit more effector cells to the site of infection, and pro-inflammatory cytokines like TNF-α and IL-1α to enhance the

inflammatory response of the tissue and innate immune cells, inducing fever, AMP production and immune cell activation (Dias *et al*., 2008). Later in the infection neutrophils will upregulate anti-inflammatory cytokines like Tumour growth factor β1 and IL-1 receptor agonist which suppress the action and recruitment of effector cells in order to inhibit inflammatory damage. Neutrophils also upregulate tissue repair factors like vascular endothelial growth factor and tumour growth factor α to induce angiogenesis, matrix invasion and cell proliferation within tissues damaged during the infection (Tecchio *et al*., 2014).

1.4.4 Neutrophil Phagocytosis

Phagocytosis is the process by which neutrophils (and other professional phagocytes) bind to, engulf, isolate and kill pathogens such as UPEC. The process of phagocytosis in neutrophils is significantly enhanced by the opsonisation of the target particle (Katzenmeyer *et al.*, 2017). Common opsonins include complement proteins produced in the liver and antibodies produced by other components of the immune system, but research has revealed that neutrophils are also capable of releasing their own opsonising proteins into the inflammatory space (Torabi and Fantone, 1990).

Stimulation of FcγR or CR by pathogenic motifs at the surface of the neutrophil will trigger the transduction of a signal into the cell, resulting in extensive rearrangement of the actin cytoskeleton so that the triggering particle is engulfed within a membrane-bound vesicle known as the phagosome (Lee, Harrison and Grinstein, 2003). Depending on which receptor is stimulated, different signalling cascades are triggered resulting in slightly different methods of phagocytosis. CR-mediated phagocytosis is slower and greatly aided by priming whereas FcγR mediated phagocytosis is quicker and more aggressive (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002). Should both FcγR and CR be activated then the two receptors will work in tandem, greatly enhancing phagocytosis (Uribe-Querol and Rosales, 2020). FPRs are also thought to have roles in phagocytosis but are not essential to the process (Weiß *et al*., 2019).

1.4.5 Neutrophil Granules

Following phagocytosis of a UPEC the neutrophil will mobilise numerous granules to the membrane of the phagosome which fuse and release their contents. Neutrophils have four distinct classes of granules. Primary (or Azurophilic) granules are formed in the earliest stages of neutrophil differentiation. These are the largest granules and serve as the primary storage site for bactericidal enzymes such as neutrophil elastases and serine proteases, and

AMPs such as cathepsins and defensins. Being the largest granules, they are also the slowest to be mobilized following the formation of the phagosome. Secondary (or specific) granules are the second to be formed, the second largest and the second slowest to be mobilized. Secondary granules contain more antimicrobial compounds, most of which are dedicated to inhibiting bacterial growth, iron sequestration or adhesion as opposed to direct killing. Examples include lactoferrin and calprotectin. Tertiary (or gelatinase) granules are formed in the later stages of neutrophil differentiation as the cell is fully maturing. They are smaller than the previous two granules and thus quicker to be mobilised. These granules are less relevant in the context of bacterial destruction and instead contain extracellular membrane-degrading enzymes such as Metallomatrix proteinase 9 (MMP9) and gelatinase which are critical in neutrophil extravasation (Borregaard and Cowland, 1997). Secretory vesicles (SVs) are the fourth type of granule. These SVs are not limited to being formed during differentiation and are constantly being processed by the trafficking machinery within the cell. These vesicles are responsible for the delivery of enzymes and channels to the membrane of the phagosome (DeLeo *et al.*, 1999). All four classes of granule are also trafficked to the surface of the neutrophil, where either the granule contents or sometimes whole granules are projected into the extracellular space (Othman *et al.*, 2022; Marki and Ley, 2022).

1. 5 The Oxidative Burst

1.5.1 Reactive Oxygen Species

Among the many enzymes and proteins delivered to the phagosome by secretory vesicles, perhaps the most important are the components of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2), an enzyme complex responsible for a key method of neutrophil killing, the generation of reactive oxygen species (ROS), also known as the oxidative burst (Bedard and Krause, 2007).

Reactive oxygen species are highly reactive molecules formed from the partial reduction of molecular oxygen. ROS is an umbrella term for various radical and non-radical species which can all be formed during reactions with oxygen including the hydroxyl radical ($^{\bullet}OH$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are prevalent in biological reactions due to the abundance of oxygen, indeed within any redox reaction involving oxygen there is the potential for ROS to be generated. As such there are a multitude of reactions in the body which can generate ROS as a byproduct or an intermediate, but in some cases, ROS are also produced deliberately (Zhang *et al.*, 2016). The oxidative burst is

the process by which phagocytes such as neutrophils enzymatically generate ROS via the NOX complex, which accumulate at high concentrations within the phagosome and can damage and kill any engulfed pathogens including UPEC. ROS can also be released into the neutrophil surroundings by NOX2 at the cell membrane.

1.5.2 NADPH oxidase 2 (NOX2)

NOX2 is comprised of 6 subunits: two transmembrane proteins ($P22^{phox}$ and $NOX2/GP91^{phox}$) forming the catalytic ROS-producing core, three cytosolic subunits ($P40^{phox}$, $P47^{phox}$ and $P67^{phox}$) which bind as a trimeric complex to the catalytic core, and a small GTP hydrolase, either Ras-related C3 botulinum toxin substrate (Rac) 1 or 2 (Groemping and Rittinger, 2005; Hordijk, 2006).

NOX2 can be thought of as having two states; inactive, where its components are physically isolated, and active, where the complex is assembled, and any further stimulation will trigger the production of ROS. Between these two states the enzyme complex is 'primed', wherein certain signals can induce changes such as the translational or conformational changes in key proteins, translocation of the NOX2 subunits or the activation of Rac which can enhance the speed at which NOX2 assembles and the robustness with which it activates (El-Benna, Ruedi and Babior, 1994; Park and Babior, 1997; DeLeo *et al.*, 1998; El-Benna *et al.*, 2008). Priming is not necessary for the activation of NOX2 but can enhance its activation and ROS production (Walker and Ward, 1992, Hallet *et al*., 1995).

1.5.3 Formyl Peptide Receptor 1

FPR1 is an example of a receptor which can both prime and fully activate NOX2 depending on the level of stimulation (Ye *et al.*, 2009). FPR1 is GPCR comprised of a transmembrane domain which passes through the membrane seven times, the extracellular receptor region, and the intracellular domain which interacts with three G proteins, α, β and γ, where they form a trimeric complex at a resting state, and dissociate into GαI and Gβγ upon ligand binding (Rosenbaum *et al.*, 2009). The role of GαI in FPR1 driven NOX2 activation is not fully understood, however it is thought to be involved in the downregulation of second messenger cAMP which inhibits NOX2 formation (Hellstrand *et al.*, 1994). Gβγ is known to have many downstream effects which can contribute to the assembly of NOX2. Gβγ can activate phosphoinositide 3-kinase (PI3K) which catalyses the synthesis of Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), a second messenger (Camps *et al.*, 1992).

PIP_3 alongside Gβγ can activate PIP_3 dependent Rac exchange factor 1 (PREX1), a guanine exchange factor (GEF) which can bind to and activate Rac1 (Weiner, 2002). PI3K can also trigger downstream kinase cascades whose kinases can phosphorylate NOX2 subunits and GDP dissociation inhibitor (GDI), an inhibitor of Rac. Gβγ can activate phospholipase beta which catalyses the cleavage of Phosphatidylinositol (4,5)-bisphosphate (PIP_2) into Inositol trisphosphate (IP_3) and Diacyl glycerol (DAG), two more second messengers which can activate various kinases such as protein kinase C (PKC), and protein lipases D and A2, all of which are involved in the activation of NOX2 components (Watson *et al.*, 1991; Li *et al.*, 2000). IP_3 also has a role in triggering the release of intracellular calcium stores, high levels of intracellular calcium can stimulate S100A8/A9 calcium sensor proteins which interact with NOX2 subunits and activate them (Berthier *et al.*, 2003; Figure 1.2).

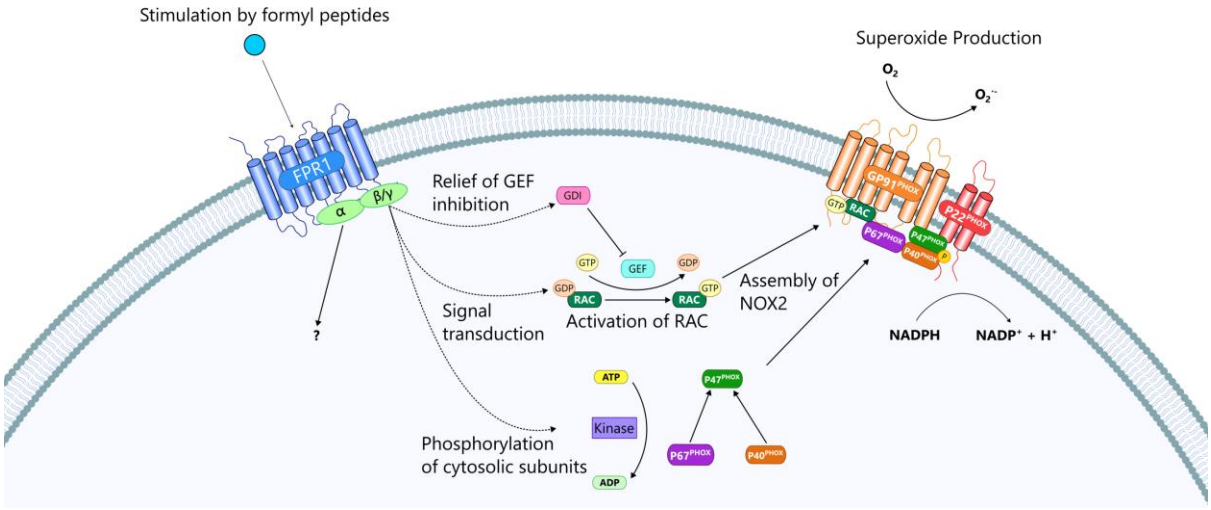

Figure 1.2 FPR1 driven NOX2 assembly.

Stimulation of FPR1 triggers the dissociation of cytosolic subunits Gα and Gβγ which trigger downstream signalling pathways leading to the activation of key kinases and second messengers. These can in turn activate the cytosolic subunits and Rac allowing them to assemble at the membrane. Once at the membrane, interactions between $P67^{phox}$ and $GP91^{phox}$, which are stabilized by the other subunits, allow for the generation of superoxide.

1.5.4 Assembly of NOX2

The ultimate goal of these signalling events is the activation and assembly of the NOX2 subunits at the membrane to enable production of ROS. Two key events are involved in the assembly of NOX2: the activation and trafficking of Rac, and the activation and trafficking of the cytosolic subunits. (Groemping and Rittinger, 2005).

In the absence of a signal small GTPase Rac is sequestered in the cytosol by Rho GDP

dissociation inhibitor. Stimulation of the neutrophil causes the phosphorylation of GDI causing it to dissociate, allowing for Rac activation and interaction with $P67^{phox}$. $P67^{phox}$ is then activated by RAC via a conformational change that enables its interactions with the catalytic core (Grizot *et al.*, 2001). The three cytosolic subunits are typically found already complexed in the neutrophil cytoplasm (Leto *et al.*, 1994). At a resting state both $P40^{phox}$ and $P47^{phox}$ are in an auto-inhibited state and $P67^{phox}$ is in an inactive state. P67 is activated by interactions with RAC which have been previously described. The auto-inhibition of $P40^{phox}$ and $P47^{phox}$ can be relieved by phosphorylation by downstream kinases and via interactions with arachidonic acid, both of which unveil small protein domains which target the subunits to the membrane (Shiose and Sumimoto, 2000; Groemping and Rittinger, 2005). Priming is thought to be important in these interactions. P38MAPK, a kinase activated by priming via TNF-α or Granulocyte-macrophage colony-stimulating factor can phosphorylate $P47^{phox}$ at Ser345 (Dang *et al.*, 2006). This region serves as a binding site for the regulatory protein Proline isomerase Pin1. Pin1 will induce conformational changes in $P47^{phox}$ which facilitate easier phosphorylation by PKC resulting in $P47^{phox}$ activation (Boussetta *et al.*, 2010). Once fully activated the cytosolic unit will move to interact with the catalytic core, stabilising it and enabling the generation of superoxide. NOX2 action is driven by two haem groups found in $GP91^{phox}$, one inside the cytoplasm and one outside, each with different redox potentials. Once the NOX2 complex is assembled, NADPH will be recruited and reduced, donating two electrons to coenzyme Flavin adenine dinucleotide (FAD; Debeurme *et al.*, 2010). FAD will then transfer electrons one at a time to the cytoplasmic haem, these electrons will be moved across the membrane onto the extracellular haem, and then each donated to a molecule of oxygen, generating two molecules of superoxide (Cross and Curnutte, 1996). Importantly the transfer of electrons between the haem groups is energetically unfavourable, meaning that it can only occur if oxygen is present as the terminal electron acceptor. This prevents electrons from accumulating within the enzyme which could damage or inactivate it (Vermot *et al.*, 2021).

1.5.5 Clinical Importance of NOX2

The importance of NOX2 in the innate immune response can be highlighted in people missing the complex. Chronic granulomatous disease (CGD) is a genetic immunodeficiency that affects 1/250,000 people, characterised by mutations in the genes encoding any of the subunits of NOX2 (Tangye *et al.*, 2020). Patients with CGD are significantly more susceptible to bacterial and fungal infections, with invasive fungal infections alone

accounting for 35% of CGD patient mortalities. CGD patients also exhibit hyperinflammation and frequently suffer from autoimmune diseases highlighting the role of ROS in immunomodulation (Marciano *et al.*, 2015).

1.6 ROS-mediated Antimicrobial Mechanisms

1.6.1 ROS Interactions with Bacteria

The superoxide radicals generated by neutrophils in contact with *E. coli* will first encounter the bacterial cell wall and plasma membrane. As a charged molecule superoxide cannot freely cross these membranes (Kehrer *et al.*, 2010). It also does not directly damage the bacterial membrane (Reis and Spickett, 2012). Superoxide can however spontaneously dismutate into Hydrogen peroxide (H_2O_2) which as a small, uncharged molecule can freely cross microbial membranes to enter the bacterial cytoplasm, though this diffusion is enhanced by the presence of porins (Bienert, Schjoerring and Jahn, 2006). Neutrophils secrete the enzyme superoxide dismutase (SOD) which enhances the conversion of superoxide to H_2O_2, elevating its effective concentration (Iversen *et al.*, 2016).

1.6.2 Protein Damage

H_2O_2 is by far the most stable and least reactive of ROS produced during oxidative burst, reacting with biomolecules such as thiols up to 4500x slower than superoxide (Reth, 2002). However, H_2O_2 can interact with redox-active metals within the cell, namely ferrous iron, to form ferric iron, the hydroxide ion (HO^-) and the highly reactive hydroxyl radical ($HO^\bullet$) via the Fenton reaction (Winterbourn, 1995). Utilising the Fenton reaction, H_2O_2 can excise iron from iron-sulphur clusters as well as iron containing co-factors and enzymes, rendering them inactive (Liochev, 1996). Many of these enzymes are essential for bacterial function and impairing their function can impact bacterial survival. For example, 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, the first enzyme in the aromatic amino acid biosynthesis pathway relies on a ferrous iron cofactor to both bind substrate and stabilise an intermediate molecule. H_2O_2 reacts with this co-factor and the resulting ferric iron will dissociate, inactivating the co-factor and by extension the enzyme (Sobota, Gu and Imlay, 2014). A similar obstruction can occur in the tricarboxylic acid cycle when H_2O_2 excises ferrous iron from the [4Fe-4S] iron-sulphur clusters found in dehydratase enzymes, inhibiting the production of ATP (Gardner and Fridovich, 1991).

The hydroxyl radical produced in the Fenton reaction can react with many biomolecules, the

only limiting factor being its short half-life which limits its diffusion; thus, it can only react with molecules in close proximity to its formation**.** In iron-containing enzymes and proteins, even if the conversion of ferrous iron to ferric iron does not inhibit function, the resulting hydroxyl radical can cleave proteins and oxidise amino acids, altering their properties. Methionine, whose sulphur group is particularly vulnerable to oxidation, loses its hydrophilic properties when oxidised which in turn can alter the folding of the protein it is incorporated into (Chao, Ma and Stadtman, 1997).

In addition, neutrophils produce the enzyme myeloperoxidase (MPO) which reacts with H_2O_2 to produce Compound I, a short-lived intermediate that can be halogenated to produce a hypohalous acid such as hypochlorous acid (HOCl; Furtmüller *et al.*, 2003; Parker *et al.*, 2011). These acids are strong oxidisers that can readily react with methionine and cysteine groups in proteins (Chapman *et al.*, 2009). These reactions disrupt protein structure and cause sulphur-containing proteins to form aggregates (Rosen *et al.*, 2009). Furthermore, HOCl can react with H_2O_2 to form singlet oxygen and peroxyl radicals, two highly reactive ROS (Miyamoto *et al.*, 2006). The reaction between HOCl and sulphur also generates toxic haloamines which break down into reactive aldehydes and ammonia, which itself can react with hypochlorous acid to form Ammonium chloride (Thomas, Jefferson and Grisham, 1982; Hazen *et al.*, 1998). This cascading effect has an immensely potent bactericidal impact; indeed, the toxic effects of MPO-derived acids have been found to be considerably higher than the contents of primary neutrophil granules (Klebanoff *et al.*, 2013).

1.6.3 Nucleotide Damage

Within bacterial cytoplasm there is a free pool of iron used in many cellular pathways. As a charged molecule iron is ‘sticky’ and tends to associate with biomolecules such as nucleic acids (Henle *et al.*, 1999). It is through these interactions that hydroxyl radicals have the opportunity to interact with nucleic acids. Oxidative stress can have a substantial impact on nucleic acids in all their forms. Hydroxyl radicals produced in close proximity to DNA can oxidise, mutate and cleave both nucleic acids and their deoxyribose linkages. 8-oxo-7,8-dihydroguanosine (8-oxo-G) is a commonly utilised marker for oxidative stress. Guanine is more easily reduced than other nucleotide bases and oxidised bases surrounding guanine can transfer an electron to it, generating 8-oxo-G (Candeias and Steenken, 1993). 8-oxo-G can base pair with both cytosine and adenine, impacting its downstream coding effect on protein translation (Cheng *et al.*, 1992). Oxidative damage to nucleotides can also impact the

transcriptional and translational machinery of bacteria. Oxidative lesions on mRNA can lead to the recruitment of non-cognate amino acids in translation (Drummond and Wilke, 2008). Work investigating the effects of oxidative stress in *E. coli* observed that oxidative lesions on mRNA interfere with its interactions to the small subunit of the ribosome, preventing it from entering an active configuration and recruiting tRNA, potentially halting translation entirely (Willi *et al.*, 2018). 8-oxo-G modifications additionally inhibit peptide bond formation in the ribosome, significantly stalling translation (Simms *et al.*, 2014).

Elongation factors (EF) are proteins which traffic tRNA and amino acids to and from the ribosome. Work in *E. coli* has demonstrated that EF-G is particularly susceptible to oxidative stress (Tamarit *et al.*, 1998). EF-G is a G protein which hydrolyses GTP for energy and is crucial to the elongation step of translation. Oxidation of EF-G by H_2O_2 causes the formation of a disulphide bridge between two cysteine residues, inhibiting EF-Gs ATPase activity and considerably slowing translation (Nagano *et al.*, 2015).

1.6.4 ROS Signalling

ROS are short-lived and highly mobile; this makes them excellent candidates for signalling molecules. ROS signalling has various immunomodulatory effects on many immune cells including neutrophils themselves. ROS are thought to hold a key role in antibacterial autophagy, wherein bacteria-laden phagosomes are matured into autophagosomes which fuse with lysosomes, destroying their contents (Huang and Brumell, 2014). The role of ROS in autophagy is not fully understood but work by Huang *et al.* (2009) demonstrated that NOX2-derived ROS are necessary for the initiation of bactericidal autophagy and observed that ROS have effects on Light Chain proteins which are critical in trafficking the phagosome to the lysosome for destruction.

ROS are also known to influence the chemotactic ability and survival of neutrophils which can help prevent excessive oxidative stress and also enhance FcγR driven phagocytosis (Pricop et al, 1999). ROS also have many effects on surrounding cells, for example, ROS can influence the polarization of macrophages, with different levels of oxidative stress inducing either inflammatory M1 polarization or repair-driven M2 polarization. ROS also contribute to macrophage phagocytosis and chemotaxis (Tan *et al*., 2016).

H_2O_2 is known to influence the expression of antigen presenting surface complexes on dendritic cells and has been demonstrated to lower their production of IL-12 which in turn inhibits T-cell maturation (Preynat-Seauve *et al*., 2003). Exposure to ROS can trigger

immature T cells to mature into regulatory T cells, immunoregulatory cells which suppress both the innate and adaptive immunity (Kraaij *et al*., 2010)

1.6.5 NETosis

Neutrophil extracellular traps (NETs) are web-like extracellular structures composed of chromatin, histones and DNA. NETs are utilised by neutrophils to trap bacteria, allowing other phagocytes to engulf them (Takei *et al.*, 1996). NETs can be formed in one of two ways, the first is a type of cell death wherein the neutrophil will decondense its nucleus and undergo controlled inflammation-driven death (pyroptosis), effectively projecting the NET into the extracellular space and releasing large quantities of pro-inflammatory molecules (Remijsen *et al.*, 2011). In comparison, vital NETosis still involves the decondensing and ejecting of nuclear material, but the cellular membrane remains unharmed, and the neutrophil can continue as an anuclear cell, still capable of phagocytosis and oxidative burst (Pilsczek *et al.*, 2010; Yipp *et al*., 2012). There are additional examples of neutrophils using mitochondrial DNA to project small fast-acting NETs into their surroundings (Yousefi *et al.*, 2009). Evidence also suggests that NETs have a direct bactericidal effect, wherein the contents of primary and secondary granules as well as ROS and AMPs are embedded in the NET structure, killing any entrapped bacteria (Brinkmann *et al.*, 2004). Emerging research has identified ROS as key regulators of NETosis. The mechanisms behind this are not fully understood, however, inhibition of NOX2 has substantial effects on the rate of NETosis (Leung *et al.*, 2021). It is thought that excessive ROS signalling may drive traffic of primary and secondary granules not only to the phagosome but also the nucleus, wherein their contents can begin the decondensing of DNA and the breakdown of the nuclear envelope (Björnsdottir *et al.*, 2015). These enzymes alongside excess ROS will also stimulate excessive DNA repair which itself is known to stimulate NETosis (Azzouz, Khan and Palaniyar, 2021).

1.7 Bacterial response to oxidative stress

1.7.1 Constitutive resistance

Microbes are constantly exposed to low levels of oxidative stress. As such bacteria have evolved a number of tolerances and resistance mechanisms to oxidative stress (Imlay, 2015). Various quality control mechanisms scan the genome of bacteria searching for oxidative damage (Kuzminov, 1999). Oxidized bases can be excised and repaired by specialised enzymes such as endonucleases (Doetsch *et al.*, 1987). Should the extent of oxidative damage

be too much for these enzymes to handle they will activate the SOS response which leads to the upregulation of genes involved in repairing DNA and arresting cell division (Maslowska *et al.*, 2019). Additionally, the bacterial cytoplasm is patrolled by various ROS-scavenging enzymes responsible for neutralizing intrinsically produced ROS. Superoxide cannot cross the cell membrane but can be produced within the cell. Superoxide dismutase, much like that produced in neutrophils is constitutively produced in bacteria and breaks down the reactive superoxide into the less reactive hydrogen peroxide (Imlay, 2013). This H_2O_2 can then be degraded by catalases and peroxidases. Peroxidases such as *E. coli's* alkyl hydroperoxide reductase (Ahp) transfer electrons from Nicotinamide adenine dinucleotide to hydrogen peroxide, converting it to water. These are expressed at high levels under low H_2O_2 stress (Seaver and Imlay, 2001). Catalases do not need an electron donor to function, instead their mechanism uses two H_2O_2 molecules, one is reduced into water and the other is used to regenerate the enzyme, as such they are only efficient under higher H_2O_2 stress (Everse, 2004). Finally, the chaperone protein HSP33 can be found patrolling the cytoplasm. HSP33 contains four Cys residues co-ordinated by a zinc and is readily oxidised by hypohalous acids. Upon oxidisation the zinc is released and the protein folds into a holdase, a chaperone responsible for binding unfolded proteins and substrates to prevent them from forming toxic aggregates (Winter *et al*., 2005).

1.7.2 Active Defence

While these mechanisms are suitable for controlling the low levels of constitutively produced ROS in bacteria, they can become quickly overwhelmed when an exogenous source of ROS is introduced such as the oxidative burst. Pathogens have developed various specialised systems to deploy under extreme oxidative stress, alongside other mechanisms to impede the oxidative burst all together (Vatansever *et al*., 2013).

OxyR is a transcription factor activated by high levels of H_2O_2 (Storz and Tartaglia, 1992). OxyR possesses a hyperreactive sensory cysteine residue that is sensitive to oxidisation by H_2O_2. Upon oxidation, proteins undergo conformational changes, swinging the residue towards another cysteine and forming a disulphide bond (Choi *et al.*, 2001). This bond locks OxyR into its active form, enabling it to recruit DNA polymerase and bind to DNA regions with an OxyR binding site. OxyR is involved in the upregulation of over twenty genes which work to endure oxidative stress (Table 1.2; Zheng *et al.*, 2001).

The SoxRS system is another oxidative stress response, however SoxRS is largely exclusive to *E. coli* (Liochev *et al*., 1999). SoxRS is composed of two proteins, the regulatory

homodimer SoxR and the transcription factor SoxS. The SoxR dimers each contain a [2Fe-2S] iron-sulphur cluster which is sensitive to oxidisation. In their oxidised form they are able to bind to DNA and induce the transcription of SoxS, SoxS is then able to induce the transcription of over 100 antioxidant genes (Table 1.2). SoxRS is an immensely potent system, allowing *E. coli* to overcome concentration of H_2O_2 10^6 times higher than usual (Zhu and Dai, 2019).

Table 1.2. Genes induced by OxyR and SoxRS

Name and gene	Function	Induced by	Citation
Neutralise ROS			
Catalases *KatG* + *KatE*	Break down H_2O_2 into water and oxygen	SoxRS and OxyR	Singh *et al*., 2008
Superoxide dismutases *SodA* + *SodB*	Neutralise superoxide into H_2O_2	SoxRS	Liochev *et al.*, 1999
Alkyl hydroperoxide reductase *Ahp*	Neutralises H_2O_2	OxyR	Seaver and Imlay, 2001
Cytochrome C Peroxidase *Ccp*	Uses H_2O_2 in the electron transport chain to generate ATP	OxyR	Khademian and Imlay, 2017
Regulatory sRNA *micF*	Downregulates porins, inhibiting H2O2 influx	SoxRS	Chou, Greenberg and Demple, 1993
Glucose-6-phosphate 1-dehydrogenase *zwf*	Crucial in the production of NADPH which regenerates antioxidant enzymes	SoxRS	Chou, Greenberg and Demple, 1993
Sequester Iron			
Ferrochelatase *HehH*	Depletes cytoplasmic iron stores by synthesising heme-containing enzymes	OxyR	Mancini and Imlay, 2015
Ferric uptake regulator *fur*	Represses iron import systems limiting the pool of cytoplasmic iron	SoxRS and OxyR	Zheng *et al*., 2001
Mini ferritin *Dps*	Sequesters free iron using H_2O_2 to catalyse the reaction	SoxRS and OxyR	Ilari *et al*., 2002
Manganese importers *MntH*	Import manganese to replace iron in metalloenzymes	SoxRS and OxyR	Anjem, Verghese and Imlay, 2009
Repair ROS Damage			
Glutathione reductase *Gsr*	Regenerates glutathione which reduces disulfide bonds formed by oxidative stress and regulates OxyR activity	OxyR	Åslund *et al*., 1999
Thioredoxin reductase *Txnr*	Regenerates thioredoxin which reduces disulfide bonds formed by oxidative stress	OxyR	Hishinuma *et al*., 2008
Endonuclease IV *nfo*	Repair oxidative damage in DNA	SoxRS and OxyR	Fawcett and Wolf, 1995
Glutathione Synthetic Enzyme *gshA*	Upregulates the production of glutathione synthesising enzymes	SoxRS	Sakamoto *et al*., 2015
Oxidative fumarase *FumC*	Maintains the citric acid cycle under iron limitation and oxidative stress	SoxRS	Himpsl *et al*., 2020

Genes induced by OxyR and SoxRS can be grouped into three major functions, the neutralisation of ROS, the sequestration of iron and the repair of ROS-induced damage (Table 1.2). Catalases, reductases and dismutases work to neutralise cytosolic ROS by breaking them down into harmless byproducts such as water and oxygen. These help to prevent ROS from accumulating to toxic levels. The depletion and sequestration of cytosolic iron aids in limiting the Fenton reaction and the production of dangerous hydroxyl radicals. Under oxidative stress *E. coli* will import manganese to be used in place of iron in metalloenzymes. Manganese works less efficiently than iron in these enzymes but is unreactive to H_2O_2. Dps is a 12-protein ring that can sequester iron into its hollow core and is upregulated by OxyR (Ilari *et al.*, 2002). To be incorporated, iron must be in its ferric form and Dps cleverly utilises H_2O_2 as an oxidiser in this conversion. Dps recruits two ferrous iron groups simultaneously, using H_2O_2 to oxidise the first iron and the second iron acts to neutralise the resulting hydroxyl radical (Williams and Chatterji, 2023). Endonucleases are crucial in the repair of oxidative damage to DNA and are highly upregulated during oxidative stress. Similarly, antioxidants glutathione and thioredoxin are crucial in the repair of oxidised cysteine residues. Importantly this includes the residues oxidised in OxyR and strain S, allowing the systems to switch themselves off after the levels of ROS become tolerable again.

1.8 Recurrent UTIs and Immune Tolerance

Recurrence is a common issue with UTIs, with 30-40% of all UTIs becoming recurrent or chronic. Not only do recurrent UTIs increase the burden on healthcare providers and the patient, but they also drive AMR (Ku *et al*., 2024).

A potential cause for the persistence of UTIs is the nature of bladder immunity itself. Inflammation, whilst important in clearing pathogens can also do substantial damage to local tissue. Reactive oxygen species released by neutrophils and toxic granules released by other leukocytes can do as much damage to the urothelium as they can to the invading pathogen. To avoid this, inflammation and the innate immune system are tightly controlled within the urogenital tract. Thus, bladder immunity is often referred to as ‘muted’ or ‘tolerant’ (Chan *et al.*, 2013). Because of their destructive mechanisms of action, the recruitment of neutrophils to the bladder is precisely controlled. Indeed, there is a direct correlation between the number of neutrophils recruited to the bladder and the bacterial burden, suggesting that the bladder does not want to ‘overcompensate’ with too many neutrophils (Shahin *et al.*, 1987). Following recruitment from the blood vessels, neutrophils reside within the connective tissue of the bladder, which they can traverse relatively freely. However, to enter the lumen of the

bladder, neutrophils must first cross the basement membrane, a dense sheet of collagen and laminin which proves to be an impassable barrier. Work by Schiwon *et al* (2014) outlines how two subsets of macrophages, the tissue-resident sentinel $Ly6C^-$ macrophages and the circulating helper $Ly6C^+$ macrophages co-ordinate neutrophil recruitment. Upon sensing infection $Ly6C^-$ macrophages will secrete the chemokine CCL2, attracting the circulating $Ly6C^+$ macrophages and causing them to exit the vasculature alongside neutrophils. Should sufficient CCL2 be produced the $Ly6C^+$ macrophages will secrete TNF-α which is detected by the $Ly6C^-$ macrophages. This causes the $Ly6C^-$ macrophages to secrete CXCL2 which will stimulate the tissue-bound neutrophils to secrete MMP9 which will cleave small holes in the basement membrane allowing neutrophils into the bladder. These cellular checkpoints serve to ensure that neutrophils are only recruited when necessary. Neutrophils are also prevented from accumulating to dangerous levels by micturition which will sweep neutrophils out of the bladder alongside bacteria (Haraoka *et al.*, 1999).

In the later stages of infection, mast cells produce large quantities of anti-inflammatory IL-10 which downregulates the production of pro-inflammatory cytokines, inhibiting neutrophil chemotaxis, and halting the intervention of the adaptive immune system, preventing the formation of memory cells which might otherwise aid in future infections. This switch seems to occur in relation the damage to the epithelial barrier and shedding of BECs (Chan, John and Abraham, 2013).

The innate immune system has a limited window to clear an infection before the body downregulates it to prevent damage. Only 20% of UTIs will resolve without extraneous treatment, suggesting that the initial innate response may not be sufficient to impede bacterial colonization, and that the adaptive immune response is either not recruited, or also insufficient (Lala, Leslie and Minter, 2024). The necessity of antibiotics in the treatment of UTIs drives the development of AMR, and UTIs represent significant portion of antibiotic prescriptions (Mulder *et al.*, 2019). As such alternative or synergistic therapies to enhance or potentially replace antibiotics are a critical area of research.

1.8.1 Immunotherapy

One promising alternative avenue for the treatment of UTIs is immunomodulating drugs which can enhance the innate or adaptive immunity's ability to clear bacteria. These can be approached in numerous ways: enhancing the recruitment of immune cells to the site of infection, inhibiting bacterial interference with immunity, suppressing anti-inflammatory signals, or enhancing the speed or robustness of immune processes such as phagocytosis.

Immunotherapy is not a novel concept, in 1927 Julius Wagener-Jauregg won a Nobel prize for the development of malariotherapy, where immunogenic malaria was used to induce a high fever which aided in the clearance of certain diseases, such as chronic syphilis (Wagner-Jauregg *et al*., 1930).

One class of immunomodulating drugs already being used in the treatment of UTIs are bacterial vaccines, inactivated bacteria or bacterial components that can sensitize the bladder immunity to bacterial antigens, allowing a more robust response upon actual infection (Mobley and Alteri, 2015). However, these drugs serve as prophylactics, useful in the prevention of UTIs but inefficient at treating patients already suffering from an infection. Drugs which target the neutrophil in the context of infection must be very carefully designed as to not induce excessive or uncontrolled ROS production as this could actively harm the patient. As such work investigating the targeted stimulation of neutrophils in the context of bacteria is uncommon. A broad search of the literature found several examples of neutrophil stimulation being used to treat infection; however, none directly targeted the oxidative burst. Work by Zhu *et al* (2024) investigated the impact of nanoimmunotherapy wherein nanoparticles were used to deliver immune mediators to sites of *Staphylococcus aureus* infection. By delivering haemoglobin to the infectious microenvironment and thus increasing its oxygenation, they were able to enhance neutrophil chemotaxis and the rate of oxidative killing in mice. Park *et al.* (2013) investigated the impact of adenosine monophosphate-activated protein kinase (AMPK) stimulating drugs on neutrophil chemotaxis and phagocytosis of *E. coli* and *S. aureus in vitro.* Observing that stimulation of AMPK both enhanced neutrophil chemotaxis and phagocytosis, whilst also interfering with bacterial inhibition of neutrophil chemotaxis. Chow *et al.* (2010) demonstrated that statins, drugs typically used in the treatment of cardiovascular disease, enhanced the production of NETs by neutrophils and enhance the clearance of *S. aureus* in mice. This is supported by Caffrey *et al.* (2017) who observed a significantly lower mortality in *S. aureus*-derived bacteraemia patients who were incidental statin users. Finally, Yang *et al.* (2019a) utilised a nanofilm of elemental Tantalum to enhance neutrophil phagocytosis, pro-inflammatory cytokine production and increase neutrophil lifespans in murine models with *E. coli* and *S. aureus.*

Drugs stimulating the oxidative burst have seen extensive research in the context of chronic diseases such as arthritis or cancer (Hultqvist *et al*., 2006; Jiang, 2023), however targeted ROS amplification in neutrophils in the context of UPEC appears to be a novel concept.

FPR1 stimulating compounds RE-04-001 and RE-04-006, developed by Pronoxis, have been demonstrated to potently enhance the activation of NOX2 *in vitro* (Lind *et al.*, 2021). Utilising the HL60 neutrophil cell line, Lind *et al.* (2021) characterized the ability of RE-04-001 to activate FPR1 at a very low concentration (~1 nM). They also observed minor chemoattractive ability and prolonged FPR1 signalling when compared to conventional activators of FPR1. This prolonged activation was surmised to be as a result of the compound's resistance to breakdown within the body and reduced activation of downstream downregulating signals when compared to other FPR1 activating molecules. Work by Cotzomi-Ortega *et al.* (2025), in collaboration with Pronoxis, investigated the role of NOX2 in UTIs *in vivo* with a murine NOX2 knockout model, observing a notable impairment in the clearance of UPEC in NOX2-deficient mice as well as increased inflammation and more extensive bladder damage, highlighting the role of ROS in immunomodulation, and posit FPR1 agonists such as the RE compounds as potential therapeutic tools in the treatment of UTIs.

1.9 Aims and Objectives

The aims of this thesis are to investigate the effects of FPR1 stimulation on neutrophils and its impact on their ability to kill UPEC. This will be achieved by accomplishing four objectives:

- Assess the oxidative tolerance of *E. coli* strains to identify a high tolerance and a low tolerance candidate.

- Assess the impact of FPR1 stimulation and bacterial co-culture on neutrophil viability.

- Explore the effects of FPR1 stimulation on the ability of neutrophils to kill *E. coli*

- Investigate the impact of FPR1 stimulation on the main bactericidal functions of neutrophils; phagocytosis, the oxidative burst and NETosis.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Media

2.1.1.1 Lysogeny Broth (LB)

LB broth medium was purchased in tablet form from Oxoid (Basingstoke, UK) and 1 tablet was dissolved for every 50 ml of distilled H_2O and autoclaved at 121 °C for 15 minutes.

2.1.1.2 LB Agar

Agar Technical (no. 3) was purchased from Oxoid (Basingstoke, UK), dissolved 1.2% w/v (12 g) in 1000 ml LB and was autoclaved 121 °C for 15 minutes.

2.1.1.3 Neutrophil Complete Media

Neutrophil complete media (0.025% FBS) was prepared by adding 12.5 µl of Foetal Bovine Serum (FBS) and 50 µl of 50 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) to 50 ml of 1x Hanks balanced salt solution (HBSS) with added $CaCl_2$ and $MgCl_2$ (Gibco, London, UK). Neutrophil complete media was made fresh for each experiment.

2.1.1.4 Columbia Horse Blood Agar.

Pre-poured and sterilised Columbia Agar plates with 5% Horse Blood were purchased from Oxoid (Basingstoke, UK).

2.1.2 Reagents

2.1.2.1 Hydrogen Peroxide (H_2O_2)

Hydrogen Peroxide (30% in water) solution was purchased from Fisher scientific and diluted in sterile dH_2O, Loughborough, UK.

2.1.2.2 Gentamicin

Gentamicin (50 mg/ml in water) was purchased from Gibco (London, UK) and was diluted to a working concentration of 200 µg/ml in sterile dH_2O.

2.1.2.3 Bovine Serum Albumin (BSA)

Albumin from bovine serum (97%) was bought as a lyophilized powder from Sigma (London, UK).

2.1.2.4 Triton X-100

100% Triton X-100 was purchased from Acros Organics (Dartford, UK) and was diluted to a working concentration of 0.1% in sterile dH_2O.

2.1.2.5 Foetal Bovine Serum (FBS)

Foetal bovine serum (Qualified) was purchased from Gibco (London, UK) and stored at -20 °C in 5 ml aliquots.

2.1.2.6 N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)

HEPES (1 M in H_2O; pH 7) was purchased from Gibco (London, UK).

2.1.2.7 Dimethyl Sulfoxide (DMSO)

100% Dimethyl sulfoxide solution was purchased from Merck (Feltham, UK).

2.1.2.8 Phorbol 12-myristate 13-acetate (PMA)

Phorbol 12-myristate 13-acetate was purchased from Merck (Feltham, UK) as a lyophilized powder and reconstituted to a stock concentration of 1mg/ml in 1 ml DMSO.

2.1.2.9 Peroxidase type II from horseradish (HRP)

Peroxidase type II from horseradish was purchased from Merck (Feltham, UK) as a lyophilised powder (5000 U/20 mg) and reconstituted to a stock concentration of 2500 U/ml by adding 10 mg to 1 ml of H_2O.

2.1.2.10 4-Aminophthalhydrazide (Isoluminol)

4-Aminophthalhydrazide was purchased from Merck (Feltham, UK) as a lyophilized powder (1 g) and reconstituted to a stock concentration of 10 mg/ml in 1 ml of 0.1 M NaOH.

2.1.2.11 N-Formylmethionine-leucyl-phenylalanine (fMLF)

FPR1 stimulating fMLF was purchased from Merck (Feltham, UK) as a lyophilized powder and reconstituted to a stock concentration of 11.43 mM in DMSO (Salamah *et al.,* 2019).

2.1.2.12 Microscopy Hemacolor®

Red and purple microscopy Hemacolor® stains were purchased from Merck (Feltham, UK).

2.1.2.13 Trypan Blue

0.4% Trypan blue in water was purchased from Invitrogen (Renfrewshire, UK)

2.1.2.14 DRAQ7™

0.3mM DRAQ7™ was purchased from Biostatus (Shepshed, UK) and diluted to a working concentration of 20 μM in FACS buffer.

2.1.3 Stock Solutions

Stock solutions were prepared using either distilled H_2O or phosphate buffered saline (PBS). Stock solutions used for microbiological work were sterilised by autoclaving.

2.1.3.1 Phosphate Buffered Saline (PBS)

Phosphate buffered saline tablets were purchased from Gibco, (London, UK).

2.1.3.2 FACS Buffer

FACS Buffer was prepared by adding 250 µg powdered sodium azide (>99.5%; Sigma Aldrich; Gillingham, UK) and 1 g of BSA to 500ml of PBS and was stored at 4 °C.

2.1.3.3 Isoluminol Buffer

Isoluminol buffer was prepared at 4x concentration by adding 70 µl of isoluminol (10 mg/ml in 0.1 M NaOH; Merck) and 70 µl of HRP (100 U/ml in water; Merck) to 860 µl of 1x HBSS. Isoluminol buffer was prepared fresh for each experiment.

2.1.4 FPR1 Stimulating Compounds

FPR1 stimulating compounds RE-04-001 and RE-04-006 were kindly provided by Pontus Duner and Peter Olofsson-Sahl at Pronoxis (Trollhatten, Sweden). The compounds were supplied as a lyophilised powder and were reconstituted in 100% DMSO to 2mM and stored in darkness as stock. Any further dilutions were made with 1% DMSO. The final concentration of DMSO in all cell work never exceeded 1%.

2.2 Bacterial Culture

2.2.1 Strain Selection

Five *E. coli* strains were selected for this work (Table 2.1). Two were model strains; K12, a non-pathogenic intestinal strain, and CFT-073, a pathogenic urinary strain. Three more strains were selected from a collection of clinical isolates provided by the Hywel Dda Health Board, each of these strains were isolated from the blood of bacteraemia patients and had been genotyped in previous work (Wilkinson, 2022). Clinical strains were selected based on the presence or absence of important ROS-related resistance genes (Table 3.1).

Table 2.1. Studied *E. coli* strains

Strain	Comments	References
K12	Model laboratory strain. Non-pathogenic Intestinal strain	(Bachmann, 1972) (Gray and Tatum, 1944)
CFT-073	Model pathogenic urinary strain	(Mobley *et al.*, 1990)
63	Missing no assessed oxidative stress genes compared to CFT-073	Microbiology and Infectious Disease (MID) archive
73	Missing OxyR_1, an OxyR analogue thought to be functionally similar to OxyR	MID archive
84	Missing SoxS and SoxR	MID archive (Chick, 2020; Wilkinson, 2022)

2.2.2 Routine Bacterial Preparation.

Bacterial Cryobead (Qiagen, Manchester, UK) stocks of the relevant strains were collected from the MID archive within the Swansea University Medical School. Bead stocks were collected from the -80 °C freezer (TSX Series, Thermo Fisher, Altrincham, UK) and thawed until a bead could be removed with an inoculation loop and streaked onto Columbia horse blood agar (Oxoid). Plates were incubated overnight at 37 °C in a GS biotech incubator (Ayr, UK) and stored at 4 °C for further use. Sub-culturing was performed every two weeks by transferring a single colony to a fresh blood agar plate.

Working *E. coli* liquid cultures were prepared by taking one colony from an agar plate, inoculating it in 5 ml of LB broth and incubating overnight at 37 °C in a Kuhner Shaker X (Newport Pagnell, UK) at 200 rpm.

2.3 Hydrogen Peroxide Disc Diffusion Assay

Resistance to hydrogen peroxide in each *E. coli* strain was initially assessed with a disc diffusion assay. An overnight working culture of each *E. coli* strain was prepared (section 2.2.2) and corrected to an OD_{600} of 0.05 with a Jenway 7200 spectrophotometer (Dunmow, UK) using LB broth. A cotton swab was dipped in liquid culture and used to spread the culture evenly over LB agar plates. Plates were rotated 90 degrees and swabbed again to ensure a full coverage. Then, 6 mm discs cut from filter paper were infused with 10 µl of hydrogen peroxide at a range of concentrations (1%, 3% and 30% vol/vol in water) and

allowed to dry in darkness. The infused discs were pressed firmly onto the bacteria-swabbed plates with sterile forceps in triplicate and the plates were incubated overnight at 37 °C. Following incubation, zones of inhibition were recorded as the diameter in cm between the two farthest points through the central disc. Any abnormal projections from the zones were ignored. If zones significantly overlapped or were deformed, a radius was measured in a clear region and the result doubled.

2.4 Bacterial Inhibitory Growth Curves

To investigate the impact of ROS on *E. coli* at lower and more biologically relevant concentrations of H_2O_2, a series of growth curves were employed to determine the minimum inhibitory concentration (MIC).

An overnight working culture of each *E. coli* strain was prepared (Section 2.2.2) and corrected to an OD_{600} of 0.1, and then further diluted (1:100) in fresh LB. A 96-well Nunc™ plate (Thermo Fisher Scientific) was loaded with 100 µl of each *E. coli* strain alongside uninfected control wells of LB only. Next, 100 µl of H_2O_2 (diluted at 2x concentration in LB in a range of 0.01 to 0.1%) was loaded into wells including an untreated control. The plate was sealed with a gas-permeable membrane and incubated at 37°C in a BMG Labtech FLUOstar Omega plate reader (Aylesbury, UK). Optical density was read at 600 nm every 10 minutes, with the plate shaking before every reading, for 24 hours. Data was graphed and the lag time for growth at the first concentration above the MIC was measured when it surpassed an OD_{600} of 0.1 to the nearest hour.

Inhibitory growth curves were also used to determine if the FPR1 stimulating compounds had any direct impact on bacterial viability. Here, 100 µl of the compounds were serially diluted in LB in a 96-well Nunc™ plate (2x concentration at a range of 200 nM to 12.5 nM), an untreated bacteria-only control and a vehicle control of 100 µl 1% DMSO were also included. Following this, wells were inoculated with *E. coli* and the plate was incubated as described above.

2.5 *Ex-vivo* neutrophil work

2.5.1 Collection of whole blood

Venous whole blood was collected from healthy volunteers at the Health and Wellbeing Centre at Swansea University. Ethical approval was gained for the collection of blood by a trained phlebotomist and consent was given by every participant. Ethical approval was granted by the Swansea University Medical School Research Ethics sub-committee (Ref #

2022-0029). Blood was collected into 9ml green-top heparin tubes and used on the day of donation.

2.5.2 Neutrophil Isolation

Neutrophil isolation and all further neutrophil work was performed in a sterile biological safety cabinet (Guardian MSC T1800, Monmouth Scientific, Somerset, UK). Neutrophils were isolated from whole blood using MACSxpress® Whole Blood Neutrophil Isolation Kit (Cat: 130-104-434, Miltenyi Biotec, Woking, UK) and a MACSxpress® Separator (Miltenyi Biotec) according to the manufacturer's instructions. To prevent iron-rich erythrocytes from interfering with further study of ROS, the isolated fraction was additionally subjected to a MACSxpress® Erythrocyte Depletion Kit (Cat: 130-098-196, Miltenyi Biotec) as per the manufacturer's instructions. Following isolation, the cell fraction was washed with warm 1x HBSS and centrifuged at 300 xg for 10 minutes in a Megafuge 16R Centrifuge (Thermo Fisher). The supernatant was discarded, and the cell pellet was resuspended in 1ml of 1x HBSS. Cell viability was assessed by combining 10 µl of cell suspension with 10 µl of Trypan blue and loading 10 µl of this mix into a Countess™ 3 Automated cell counter (Thermo Fisher). Neutrophils were then diluted to the desired concentration for further work in neutrophil complete media (section 2.1) using the following calculation:

$$\frac{Cells/ml}{Desired\ cells/ml} = Dilution\ Factor\ (DF)$$

$$\frac{Desired\ volume\ (ml)}{DF} = X\ ml\ cell\ suspension$$

$$Desired\ volume\ (ml) - X = Y\ ml\ neutrophil\ complete\ media$$

2.6 Isoluminol Assay

Isoluminol is a hydrophobic chemiluminescent dye which cannot cross cell membranes. This means it can be used to measure the production of extracellular ROS in neutrophils. To begin, neutrophils were diluted to $2x10^6$ cells/ml in neutrophil complete media (section 2.1.1.3). Next, 50 µl of diluted cells (100,000 cells total) were pipetted into a white 96-well plate (Greiner, Stonehouse, UK) in triplicate, followed by 25 µl of isoluminol buffer (section 2.1). The plate was then placed in a 37 °C incubator for 10 minutes. Following incubation, cells were rapidly treated with 25 µl of 10 µM RE-04-001, RE-04-006 or fMLF. The plate was then loaded into a FLUOstar Omega luminescence reader pre-warmed to 37 ºC, and luminescence was measured every 20 seconds for 7 minutes.

2.7 Neutrophil and Bacterial Co-Culture

2.7.1 Co-Culture

To investigate interactions between stimulated neutrophils and bacteria, a standardized co-culture was required. To make an individual bacterial inoculum, first an overnight working culture of *E. coli* was prepared (Section 2.2.2) and corrected to an OD_{600} of 0.1 with HBSS. Next, the culture was centrifuged at 11269 xg (12,000 rpm) for 5 minutes in an Eppendorf Centrifuge 5425 R (Stevenage, UK) and the supernatant discarded. The pellet was resuspended in 100 µl of FBS to opsonise bacteria and incubated at 37 °C for 10 minutes. The sample was again centrifuged at 12,000 rpm for 5 minutes, the supernatant discarded, and the pellet was resuspended in 100 µl of HBSS (~$1x10^7$ bacteria). Next, isolated neutrophils (Section 2.5.2) were diluted to $1x10^6$ cells/ml and 1ml of cell suspension was loaded in each well of a 24-well plate (~$1x10^6$ cells per well). Following this 100 µl of the *E. coli* inoculum was pipetted into each well and swirled to mix giving a multiplicity of infection of 10:1. Treatments were applied to selected wells, these were 10 µM RE-04-001 or RE-04-006 10 µM fMLF or 1% DMSO. The final concentration of DMSO in each well was always ≤1% which did not affect cell viability or function. The plate was again swirled to mix and then incubated for 45 minutes at 37 °C, 5% CO_2 in a Sanyo MCO-18AIC CO_2 incubator (Bracknell, UK).

2.7.2 Gentamicin Protection Assay

A gentamicin protection assay was used to investigate any changes in phagocytosis and killing ability in FPR1-stimulated neutrophils (Figure 2.1). Following incubation (described in 2.7.1), the samples were centrifuged at 1000 xg for 5 minutes in an Eppendorf 5810 R centrifuge. Supernatants were removed carefully with a Pasteur pipette and stored on ice for later examination. The pellet was washed and resuspended in PBS twice, centrifuging between each wash. Next, 2 ml of 200 µg/ml gentamicin in PBS was added to each well and the plate was incubated for 15 minutes at 37 °C, 5% CO_2. The plate was once again centrifuged at 1000 xg for 5 minutes and the supernatant was carefully removed and discarded. This was followed by two more washes with PBS, again centrifuging between each wash. To lyse the neutrophils 1 ml of 0.1% Triton X-100 in PBS was added to each well and the plate was incubated for 10 minutes at 37 °C. Twenty µl of the lysed suspensions were serially diluted in PBS ($1x10^{-1}$ to $1x10^{-3}$) in a 96-well plate, and the previously saved supernatants were also serially diluted in PBS ($1x10^{-1}$ to $1x10^{-8}$) in a 96-well plate. Ten µl aliquots of each dilution were pipetted in duplicate onto LB agar and the plates were

incubated overnight at 37 °C. The next morning colonies were counted and used to calculate the colony forming units (CFU) per ml. The final percentage of bacteria compared to the original inoculum was then calculated using the following equation:

$$\frac{final\ CFU/ml}{10000000\ (initial\ inoculum\ CFU/ml)}\ \text{x}\ 100$$

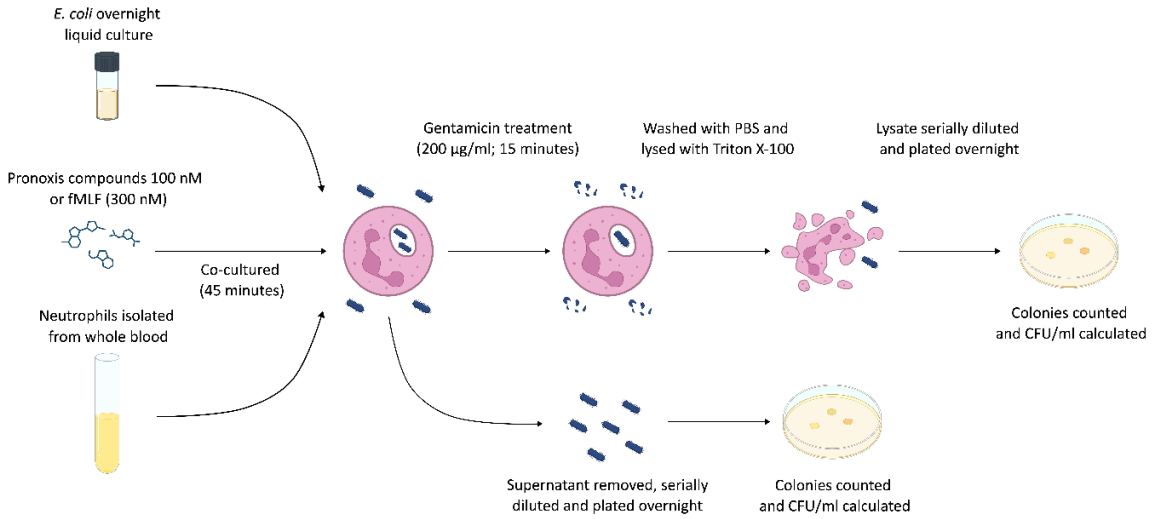

Figure 2.1 Gentamicin protection assay

Neutrophils isolated from whole blood were co-cultured with *E. coli* and FPR1 stimulating compounds for 45 minutes allowing bacteria to be phagocytosed and killed. Following incubation the supernatants were removed, diluted and plated which was used to assess the extracellular impact of FPR1 stimulated neutrophils on bacteria. Extracellular *E. coli* not collected in the supernatant was killed with gentamicin and the neutrophils were lysed, releasing intracellular *E. coli* which was then diluted and plated to assess intracellular killing.

2.7.3 Cytospin Slide Preparation

To investigate the impact FPR1 stimulating compounds had on neutrophil phagocytosis, light microscopy was used. Cytospin cartridges were assembled by inserting a glass microscopy slide into a CytoClip funnel clip (Thermo Shandon, Runcork, UK), followed by a Fisherbrand filter card, and then a CytoSep™ funnel (Thermo Shandon). A co-culture was prepared as described in 2.7.1 and following a 45-minute incubation the contents of each well were mixed by pipetting and down to resuspend any settled neutrophils. Then, 80 µl of each sample was

pipetted into the funnel of a Cytospin cartridge and the cartridges were centrifuged at 300 rpm for 3 minutes in a Shandon Cytospin 3. The cartridges were carefully disassembled, and slides were left to dry overnight. Once dry, samples were fixed with 100% methanol, stained with Red Microscopy Hemocolor (cytoplasmic and granular stain), Purple Microscopy Hemocolor (nuclear stain) and washed 2x with distilled water for 30 seconds each. Slides were again allowed to dry overnight and then cover slips were mounted with DPX mountant.

2.7.4 Phagocytosis Analysis

Slides were examined with a Nikon eclipse 50i at 1000x magnification under oil immersion. Neutrophils were identified via their distinctive multi-lobed nuclei, and bacteria were identified as small dark rods (Figure 2.2). Neutrophils were counted and noted as either having phagocytosed, where a bacteria could be seen within the cytoplasm, or non-phagocytosed, where bacteria were not seen within the neutrophil. Then, neutrophils were counted in a random field until 100 total cells had been identified and a calculation was performed to determine the percentage of neutrophils that had phagocytosed any bacteria:

$$\frac{Number\ of\ phagocytosed\ neutrophils}{100\ (total\ number\ of\ neutrophils)}\ x\ 100$$

If less than 100 neutrophils could be found on the entire slide but the number of neutrophils was close to 100 (>70) then the slide was included, and the calculation was adjusted. Slides with fewer than the 70 cells were discarded.

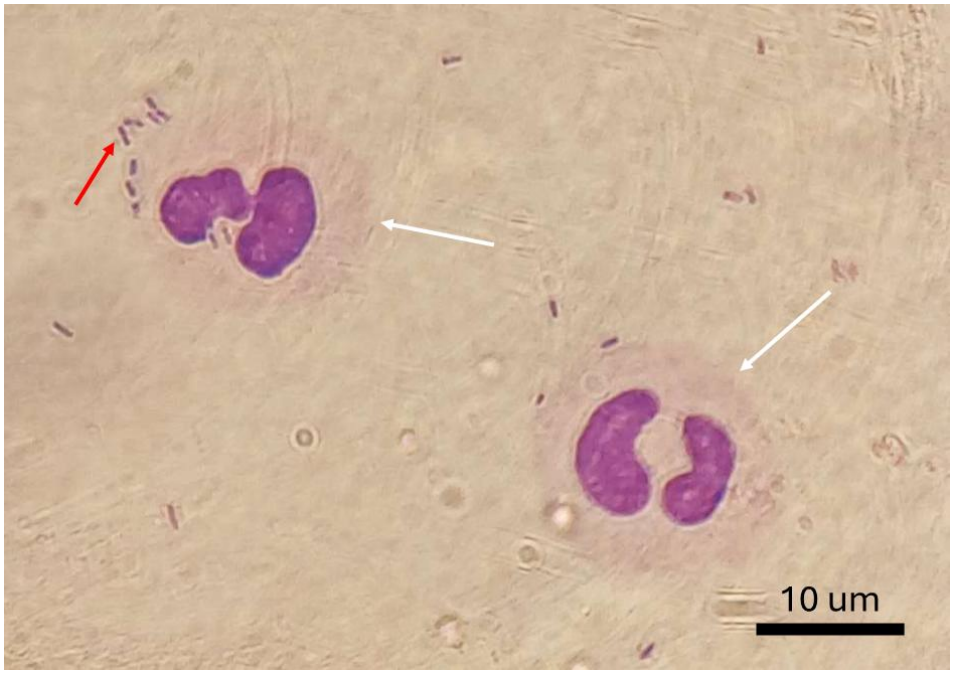

Figure 2.2 Identifying phagocytosis in neutrophils.

Two neutrophils, identified by their distinct multi-lobed nuclei can be seen marked by the white arrows. Bacteria can be seen as small dark rods; an example is highlighted by the red arrow. The neutrophil on the left has two bacteria within its cytoplasm and would be counted as having phagocytosed. The neutrophil on the right has two bacteria at the cell surface but none within the cytoplasm and would be counted as not phagocytosed.

2.7.5 Bacterial Viability Assay

A co-culture was prepared as (section 2.7.1) in individual 1.5 ml Eppendorf tubes loaded into a Fisherbrand Multi-purpose Tube Rotator and incubated at 37 °C at 10 rpm. Twenty µl of the initial inocula used to generate the co-culture (section 2.7.1) were serially diluted ($1x10^{-1}$ to $1x10^{-8}$) in a 96-well plate. Then, 10 µl aliquots were taken and pipetted in duplicate onto LB agar which was then incubated overnight at 37°C to determine the CFU/ml of the starting inoculum.

At 1-, 2- and 3-hours post-inoculation, 20 µl was taken from each sample and serially diluted ($1x10^{-1}$ to $1x10^{-8}$) in a 96-well plate in PBS. Then, 10 µl of each dilution was plated in duplicate on blood plates and the plates were incubated overnight at 37 °C.

On the following day colonies were counted and used to calculate the CFU/ml of each sample, and the data was processed with the calculation seen (Section 2.7.2).

2.8 Flow Cytometry

2.8.1 Sample Preparation

Flow cytometry was used to determine if stimulating FPR1 using the Pronoxis compounds in neutrophils impacted their viability. DRAQ7™ is a membrane impermeable fluorescent dye that stains DNA making it a suitable marker for cell death in permeabilised cells. To prepare samples including neutrophils alone: neutrophils were isolated (section 2.5.2) and diluted to $1x10^6$ cells/ml. From this, 100 µl of cell suspension was loaded into round-bottom polypropylene FACS tubes in duplicate. Select tubes were then treated with FPR1 activating compounds to one of four concentrations (25 nM, 50 nM, 75 nM, 100 nM), 300 nM fMLF or 1% DMSO. Also included were unstained, untreated, stained T_0 (kept on ice during the incubation period) and 1% Triton X-100 controls (dead cell control). Following treatment, the tubes were incubated at 37 °C in 5% CO_2 for 45 minutes. DRAQ7™ dye was diluted 1:15 from stock (from 0.3 mM to 20 µM) and 5 µl was added to each sample excluding the unstained control. Samples were stored in darkness for 10 minutes at room temperature, after which they were ready to be analysed.

To prepare samples including neutrophils and bacteria an additional series of washing steps was included. A co-culture was prepared (section 2.7.1) and 500 µl of each sample was pipetted into FACS tubes in duplicate. These were centrifuged at 500 xg for 7 minutes at 4 °C in an Eppendorf 5810 R centrifuge, the supernatant discarded, and the pellet resuspended in 100 µl FACS buffer. These samples were then stained with DRAQ7™ for 10 minutes in darkness as described above. Following incubation 2ml of FACS buffer was added to each tube and centrifuged at 500 xg for 7 minutes at 4 °C. This process was repeated and then the pellet was resuspended in 100 µl of FACS buffer leaving samples ready for analysis.

2.8.2 Sample Analysis

Samples were vortexed and then analysed with a Novocyte NovoSampler Pro (Stockport, UK). Samples were measured at a medium flow rate (35 µl/min, Core Diameter 12.2um) with collection conditions of 12,000 ungated events or 50 µl volume. The flow cytometer measured forward scatter (FSC), side scatter (SSC) and DRAQ7 fluorescence was detected using the R780 channel.

2.8.3 Data Processing

Flow cytometry data was processed using FlowJo v10 software (BD Biosciences). Data first underwent doublet discrimination to isolate the population of single cells and cellular debris. Next, a gating strategy was developed to isolate and determine the percentage of neutrophils in the population, neutrophils were identified by their large size (measured by FSC) and high granularity (measured by SSC). A triton control was used to identify the dead population and generate a gate for DRAQ7+ cells which was then applied to all other samples (Figure 2.3). Samples co-cultured with bacteria underwent an additional gating process to identify unique sub-populations (Figure 2.4).

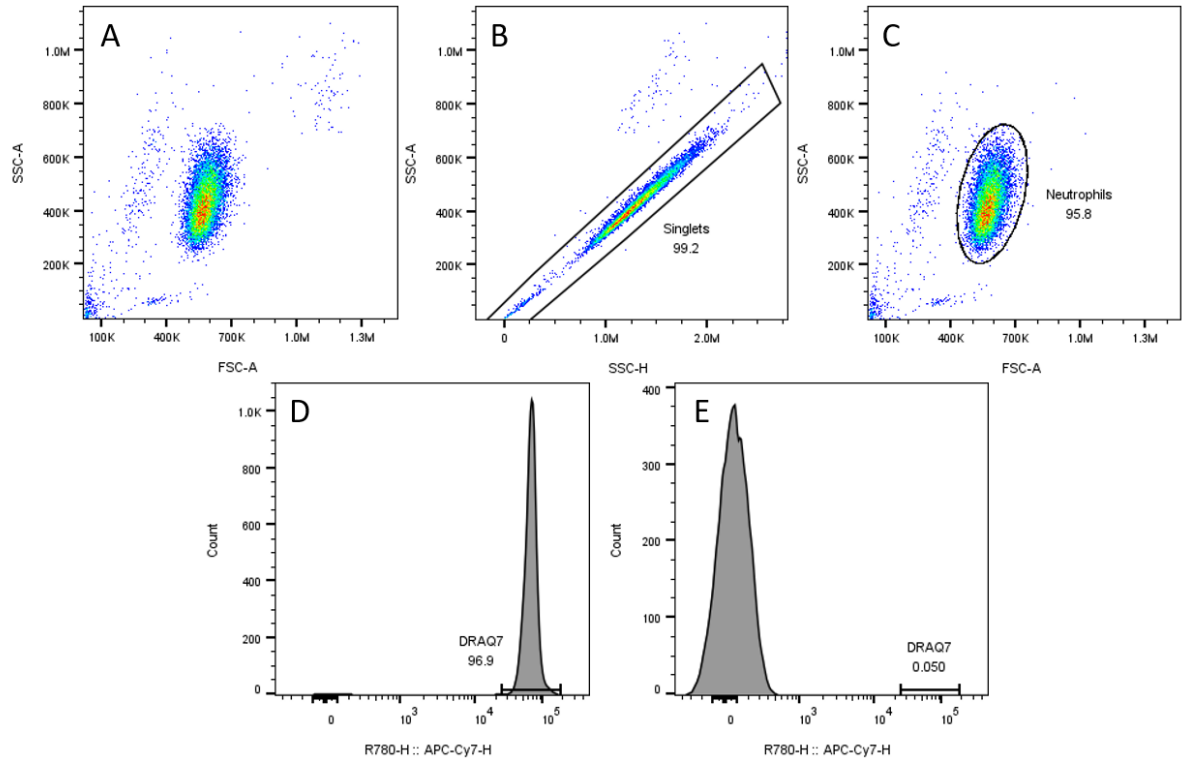

Figure 2.3. Neutrophil flow cytometry gating process.

A. An unmodified intensity plot of FSC-Area against SSC-Area. B. Doublet discrimination, an intensity plot of SSC-Area against SSC-Height plots single cells in a distinct line allowing them to be gated from doublets. C. An intensity plot following doublet discrimination with a gate applied to the neutrophil population. D. A histogram of a sample treated with Triton X-100, plotted as fluorescence intensity through the R780 channel against a frequency distribution of the data. The high fluorescence peak identifies this sample as highly DRAQ7+ indicating high cell death and a gate of this peak can be applied to all other samples. E. A histogram of a regular stained sample with the DRAQ7+ gate applied.

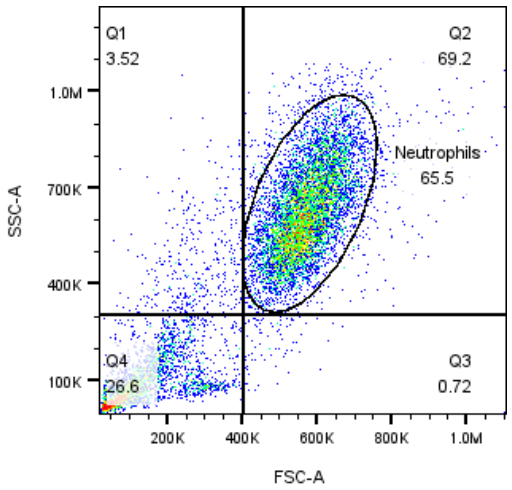

Figure 2.4 The gating process applied to *E. coli* infected samples.

Infected samples first underwent the gating process seen in Figure 2.3. The sample was then gated into quarters. Q1 gates cells with a lower FSC than gated neutrophils but a similar SSC. The percentage of gated neutrophils was subtracted from Q2 to identify events with a larger SSC or FSC. Q4 identified events with low FSC and SSC alongside cellular debris. Q3 saw minimal change between samples and thus was ignored.

2.9 Confocal Microscopy

Confocal microscopy was used to investigate the presence of NETs in stimulated neutrophils. Neutrophils were isolated (Section 2.5.2) and diluted to $2.5x10^6$ cells/ml. From this, 100 µl of diluted cells (250,000 cells) were loaded into the wells of a glass-bottomed 96-well (Greiner) plate in triplicate. The plate was incubated for 1 hour at 37 °C in 5% CO_2 allowing the neutrophils to adhere to the bottom of the wells. *E. coli* inocula were prepared (Section 2.2.2), however they were corrected to an OD_{600} of 0.01, and resuspended in 50 µl of FBS and HBSS respectively. Half of the plate was infected with 50 µl of the *E. coli* inoculum and treatments of 100nM RE-04-001, RE-04-006 or 3 µl 1µg/ml PMA (to 30 ng/ml) were applied to selected wells.

The plate was then incubated for 3 hours at 37 °C, 5% CO_2. Following incubation, the plate was centrifuged at 300xg for 5 minutes in an Eppendorf Centrifuge 5425 R, the supernatant was carefully removed and discarded, and 50 µl of 10% paraformaldehyde was added to each well. Cells were fixed for 10 minutes before being centrifuged again at 300 xg for 5 minutes, supernatant was removed and discarded, and each well was washed with 50 µl of PBS, followed by another spin. Then, each well was stained with 50 µl of 10 mg/mL DAPI in darkness for 15 minutes, followed by a final centrifugation as described above and cells were

resuspended in 50 µl of PBS, wrapped in parafilm and foil and stored at 4 °C until confocal analysis.

Samples were analysed with a Zeiss LSM 980 Confocal laser scanning microscope using Zen software. Images were taken using a x63 lens and z-stack imaging was performed with a 0.6 µm step size. Image resolution was 512 pixels^2 and the image size was 135 µm^2. Images were analysed and processed using ImageJ software (National Institute of Health, Maryland, USA).

2.10 Statistical Analysis

Statistical analysis was performed in Graphpad version 9 (La Jolla, California, USA). All data sets were tested for normality with a Shapiro-Wilks normality test or assumed not normally distributed where the test was not possible ($n < 4$). Normally distributed data was analysed with a parametric T-test or a one-way ANOVA with a Dunn's multiple comparison test. Non-normal data was analysed with a Mann-Whitney-U test or a Kruskal-Wallis tests with Dunn's multiple comparisons included. P values <0.05 were considered significant. The specific tests applied to each set of data are confirmed in figure legends.

Chapter 3: Results

3.1 Bacterial Optimisation

3.1.1 Strain Selection

Five *E. coli* strains were selected for this work. Two were reference strains: K12 and CFT-073 (Bachmann, 1972; Mobley *et al*., 1990). Three further strains were selected from an array of clinical samples from bacteraemia patients provided by the Hywel Dda health board which had been genotyped in previous work (Table 3.1, Bold; Wilkinson, 2022). A review of literature related to ROS resistance genes in CFT-073 was undertaken and urinary origin bacteraemia strains from the clinical array were selected for the presence or absence of these respective genes (Appendix A; Table 3.1).

Table 3.1 Heatmap of oxidative resistance genes found in urinary origin bacteraemia *E. coli* strains

Gene \ Strain	55	58	59	62	**63**	65	67	69	71	72	**73**	74	75	77	78	82	**84**	85	86	87
rpoS	N	Y	Y	N	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	N	Y	**Y**	Y	Y	Y
yqhC	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	N	Y	Y
yqhD	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
ahpC	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
ahpF	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	N	Y	**Y**	Y	Y	Y
bcp	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
msrA	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
msrB	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
msrC	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
soxS	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**N**	Y	Y	Y
soxR	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**N**	Y	Y	Y
ppk1	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
katE	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
katG	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
sodA	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
sodB	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
sodC	Y	Y	Y	Y	**Y**	Y	Y	N	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
oxyR	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
oxyR_1	Y	Y	Y	Y	**Y**	Y	Y	N	Y	Y	**N**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
btuE	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
tpx	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y
gor	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y	Y	Y	**Y**	Y	Y	Y

3.1.2 H_2O_2 Minimum Inhibitory Concentration (MIC)

E. coli tolerance to H2O2 was initially tested with a disc diffusion assay at 1%, 3% and 30% H2O2 (Appendix B; Figure 4.1) however, a technique with greater sensitivity was required to examine the effect of lower concentrations of H_2O_2 on the growth of the *E. coli* strains. To further investigate the tolerance to H_2O_2 in *E. coli* at more biologically relevant concentrations, growth curves were used to establish the MIC of H_2O_2 for each *E. coli* strain using concentrations of H_2O_2 at 0.01% to 0.1% (Figure 3.1; Table 3.2; Forman *et al.*, 2016). K12 demonstrated no growth in concentrations of H_2O_2 beyond 0.03%, CFT-073 displayed no growth beyond 0.05%, whereas all other strains showed no growth beyond 0.04% H_2O_2. An elongated lag phase could be seen in all strains at the lowest tolerable concentration of H_2O_2 (0.03 or 0.04%). Clinical strain 73 (Figure 3.1D) displayed the shortest lag period, entering exponential phase after approximately 2 hours, CFT-073 and 63 (Figure 3.1B and C) at 3 hours, K12 (Figure 3.1A) at 4 hours, and clinical strain 84 (Figure 3.1E) after 9 hours (Table 3.2). K12 and 73 displayed a notable increase in growth in the presence of 0.01% H_2O_2 when compared to the untreated control.

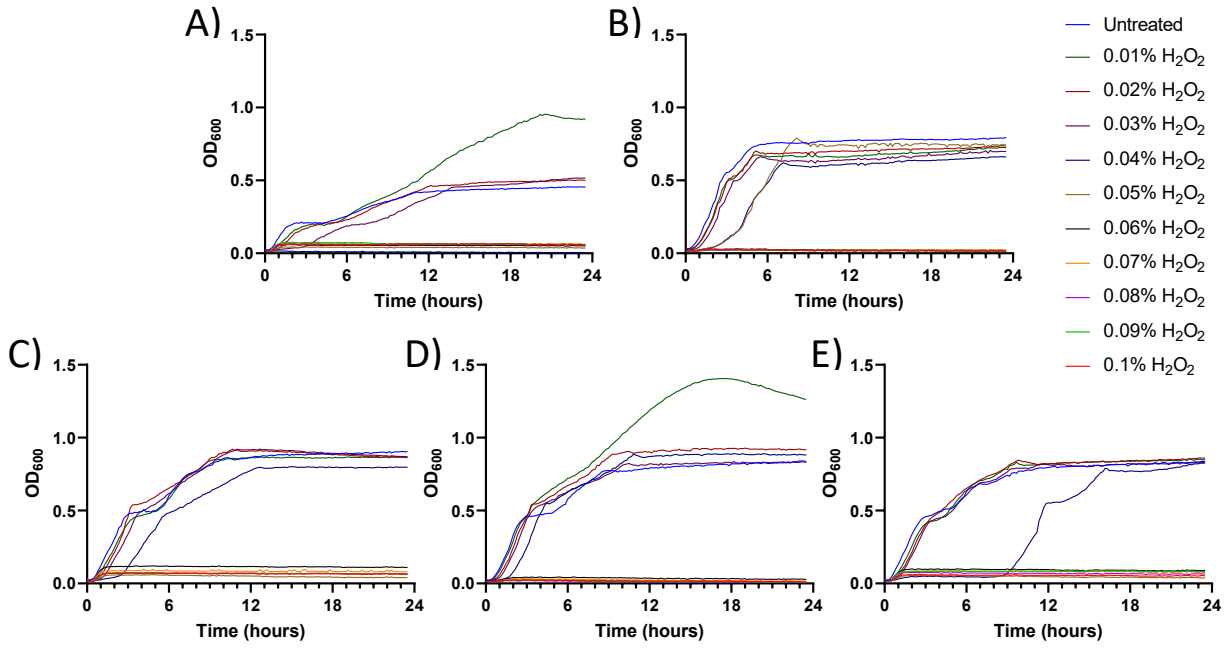

Figure 3.1. The minimum inhibitory concentration of H_2O_2 on *E. coli.*

Optical density readings of *E. coli* strains (A) K12, (B) CFT-073, (C) 63, (D) 73 and (E) 84 at OD600 over a 24-hour period. Bacteria were grown in LB broth and treated with H_2O_2 at a range of concentrations (0.1% to 0.01%). Data is from a representative experiment which was repeated 3 times.

Table 3.2. *E. coli* H_2O_2 MIC values

E. coli Strain	MIC of H_2O_2 (%)	Duration of lag phase (hours)
K12	0.04	4
CFT-073	0.06	3
63	0.05	3
73	0.05	2
84	0.05	9

3.1.3 Bacterial Tolerance to FPR1 Stimulating Compounds

To determine the impact of FPR1-stimulating compounds on microbial growth in the absence of neutrophils, *E. coli* strains were incubated with various concentrations of RE-04-001 and RE-04-006 for 24 hours (Figure 3.2). No significant effects were observed to be caused by time and strain variability, as would be expected in a standard microbial growth curve, but detected no significant effects related to treatment or treatment against time across all strains. Additionally, a Bonferroni's multiple comparisons test found no significance when comparing the mean of each sample to the untreated control in all strains. Thus, FPR1 stimulating compounds RE-04-001 and RE-04-006 at concentrations of 12.5 nM to 200 nM had no direct impact on bacterial growth, nor did the vehicle (1% DMSO).

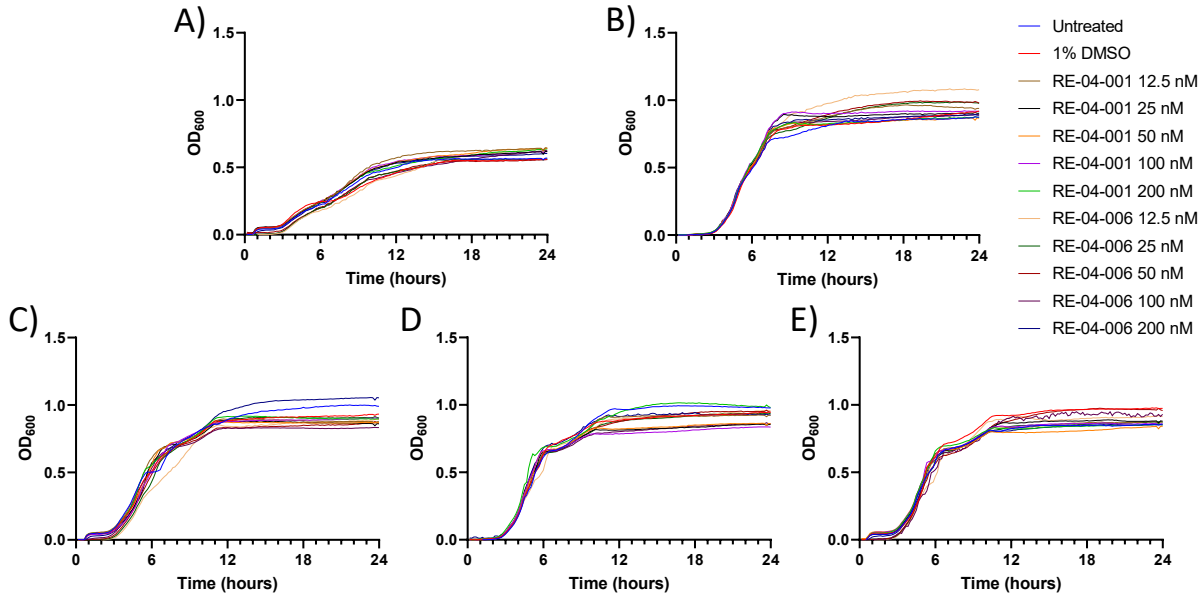

Figure 3.2 The effect of FPR1 stimulating compounds on *E. coli* growth.

Optical density readings of *E. coli* strains (A) K12, (B) CFT-073, (C) 63, (D) 73 and (E) 84 at OD_{600} over a 24-hour period. Bacteria were grown in LB broth and treated with RE-04-001 or RE-04-006 at a range of concentrations. Data points are presented as a mean (n=5). Data was analysed with a two-way repeated measures ANOVA and a Bonferroni's multiple comparisons test, but no significance was found.

3.2 Neutrophil Treatment Optimisation

3.2.1 Extracellular ROS Production

The impact of the FPR1 activating compounds on the production of extracellular H_2O_2 was measured with an isoluminol assay. Samples treated with 75 nM or 100 nM RE-04-001 (Figure 3.3A), 100 nM RE-04-006 (Figure 3.3B) and 100 nM fMLF showed a distinctive peak to roughly 12,500 RLU between 0- and 1-minutes post-stimulation. This was followed by a steady increase in luminescence up to 7 minutes. Samples treated with lower concentrations of compounds (25 nM and 50 nM) did not display this peak but followed the same steady increase in luminescence. All treated samples displayed 5-10x higher luminescence than the untreated control. Luminescence displayed by the untreated control did not surpass 5000 RLU and was defined as background luminescence. The area under the curve (AUC) was then calculated for each treatment (Table 3.3).

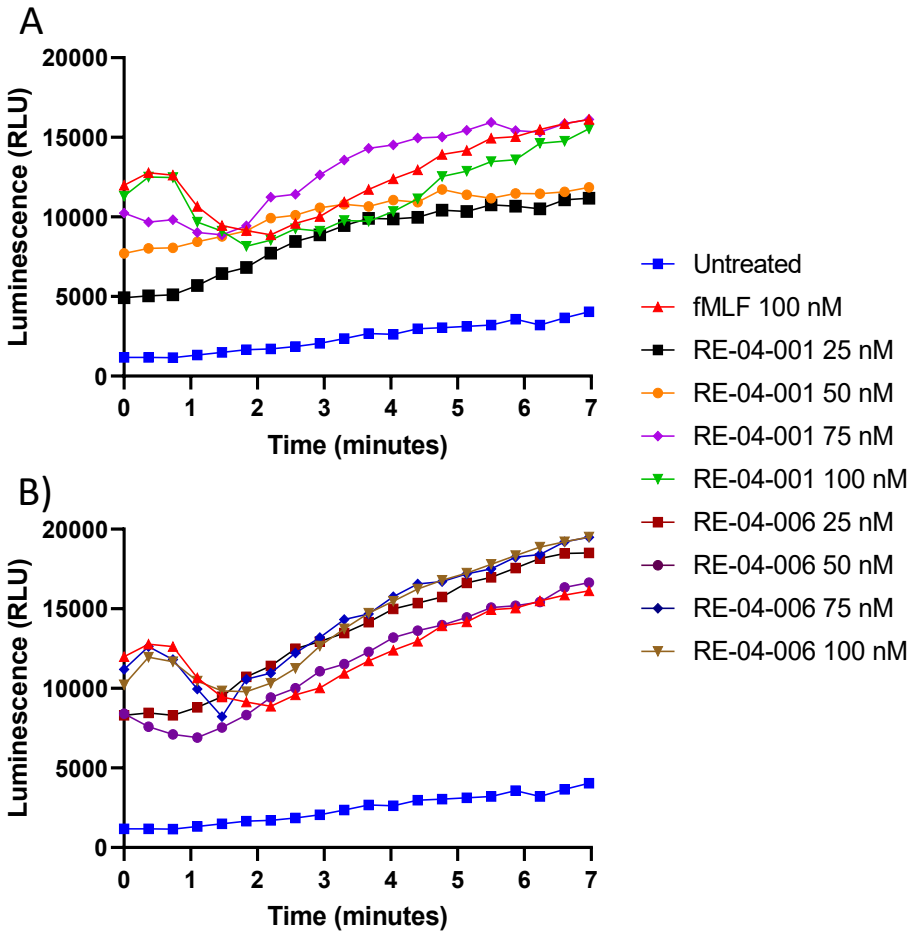

Figure 3.3 The effect of FPR1 stimulating compounds on extracellular H_2O_2 production in neutrophils.

Neutrophils isolated from whole blood were mixed with isoluminol and HRP and then treated with FPR1 stimulating compounds RE-04-001 (A) or RE-04-006 (B) at a range of concentrations to trigger an oxidative burst. Data shows luminescence by isoluminol measured every ~20 seconds for 7 minutes. n=1

Table 3.3. Area under the curve (AUC) of isoluminol assay

Treatment	AUC
Untreated	4397
fMLF 100 nM	30853
RE-04-001 100 nM	29305
RE-04-001 75 nM	29683
RE-04-001 50 nM	26249
RE-04-001 25 nM	19135
RE-04-006 100 nM	31810
RE-04-006 75 nM	32478
RE-04-006 50 nM	24437
RE-04-006 25 nM	29443

3.3 *E. coli*-Neutrophil Co-Culture

3.3.1 Intracellular and Extracellular Survival of *E. coli*

To assess the impact of FPR1stimulating compounds on the killing ability of neutrophils, a gentamicin protection assay was employed and counts in cell lysate and supernatant assessed (Figure 3.4-light purple bar comparison). The neutrophil lysates prepared after 45 minutes of co-incubation with *E. coli* were cultured on agar plates to measure bacterial counts. Across all samples K12 displayed a 2-4x higher intracellular CFU/ml after incubation with neutrophils than compared to CFT-073. Conversely, the bacterial load in supernatants, estimating extracellular *E. coli*, derived after co-incubation with neutrophils showed that the CFU/ml of CFT-073 was 2-5x greater than that of K12 samples**.** In the presence of RE-04-001, K12 counts in neutrophil lysates (Figure 3.4A) showed little difference compared to non-drug treatment (~1% increase). Treatment with RE-04-006 and fMLF elicited a much larger increase in intracellular K12, from 32,125 CFU/ml to 43,562 and 43,312 CFU/ml, respectively. CFT-073 counts from lysates of untreated neutrophils (Figure 3.4B) displayed an average CFU/ml of 11,450, treatment with RE-04-001 or fMLF resulted in a moderate decrease to 8050 and 8850 CFU/ml respectively, and treatment with RE-04-006 demonstrated a larger decrease to 4550 CFU/ml, the lowest seen across all samples. Viable K12 recovered as counts in the cell lysate (Figure 3.4C) trended towards a decrease in CFU/ml from the untreated control (812,500 CFU/ml) when treated with RE-04-001 (590,000 CFU/ml), RE-04-006 (282,500 CFU/ml) and fMLF (782,500 CFU/ml). Viable CFT-073 recorded as counts

in the cell lysate (3.5D) were increased from the average untreated CFU/ml, (4,233,333 CFU/ml), in samples treated with RE-04-001 and RE-04-006 to 4,600,000 and 5,600,000 respectively, and a decrease in samples treated with fMLF to 3,866,600.

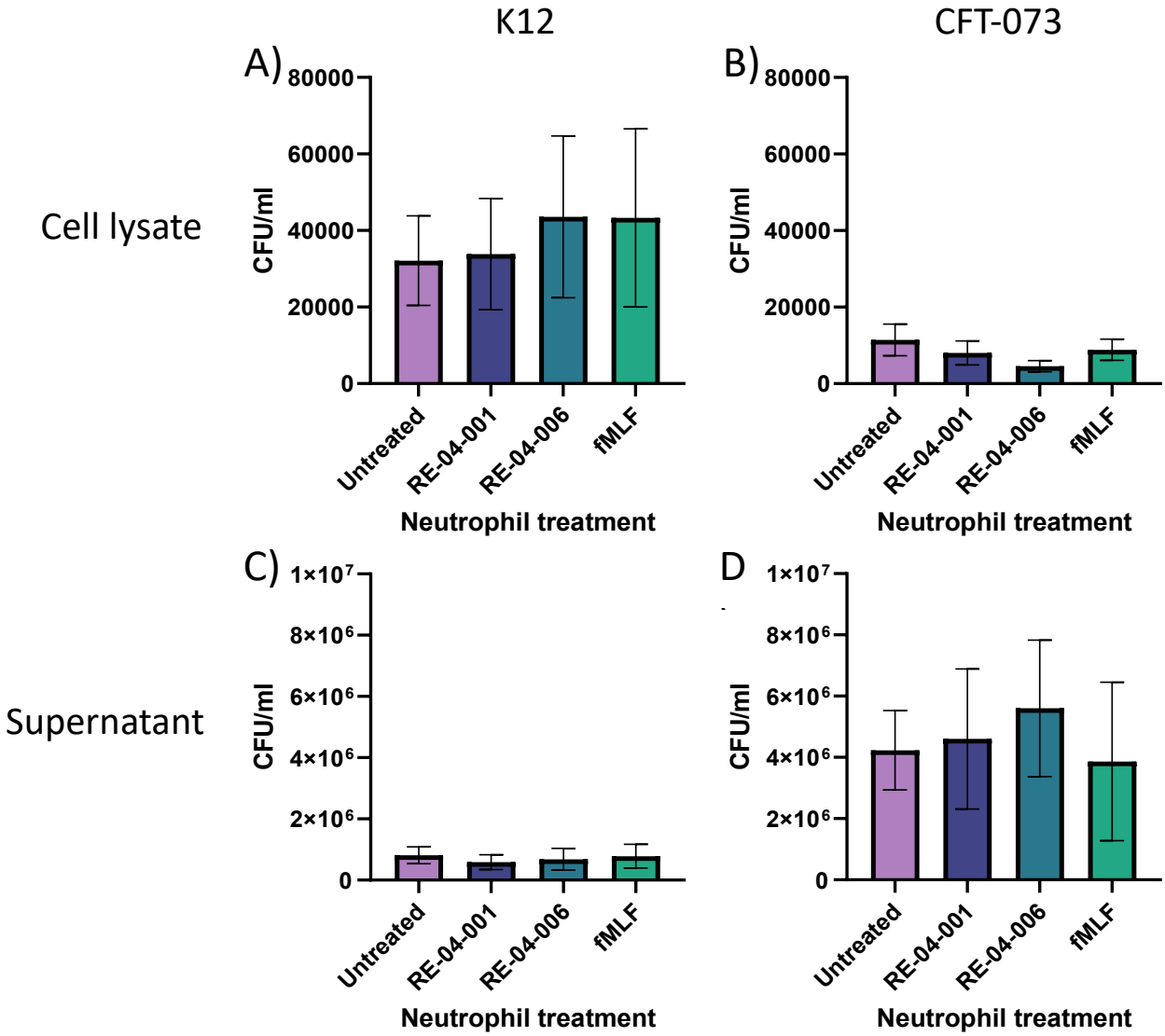

Figure 3.4. The effects of FPR1 stimulating compounds on neutrophil killing of *E. coli*
Intracellular killing of bacteria by neutrophils was assessed using a gentamicin protection assay. Neutrophils and *E. coli* strains K12 (A) and CFT-073 (B) were co-cultured for 45 minutes, and serially diluted following gentamicin treatment to calculate CFU/ml. Co-cultures with K12 (C) and CFT-073 (D) were also serially diluted without gentamicin and the CFU/ml was calculated to assess the impact of stimulating neutrophils on viable extracellular bacteria. Data is presented as a mean ± SEM (Standard error of the mean; n=4 independent donor neutrophils). Data was analysed with a one-way ANOVA and Tukey's multiple comparison test, but no significance was found.

3.3.2 Neutrophil Phagocytosis

To determine the impact of FPR1-stimulating compounds on neutrophil uptake of *E. coli* neutrophils were tested for any changes in phagocytosis following FPR1 stimulation and *E. coli* co-culture. Neutrophils co-cultured with K12 only (Figure 3.5A) showed an average phagocytosis of 46.6%, when treated with RE-04-001 or fMLF this increased by 11% and 6%, respectively. Treatment with RE-04-006 lowered the average phagocytosis of K12 by 3%. Untreated neutrophils co-cultured with CFT-073 only (Figure 3.5B) and those treated with RE-04-001 both displayed an average phagocytosis of ~76%. Neutrophils treated with RE-04-001 or RE-04-006 demonstrated a reduction in phagocytosis of 7.8% and 6.9%, respectively. No phagocytosis was observed in any uninfected neutrophils across all samples. In all samples notably more CFT-073 was phagocytosed than K12 (up to 33%). Neutrophil morphology varied heavily between donors, with some neutrophils swelling, decondensing or losing their round shape. These changes appeared independent of FPR1 stimulation (Appendix C; Figure 4.2).

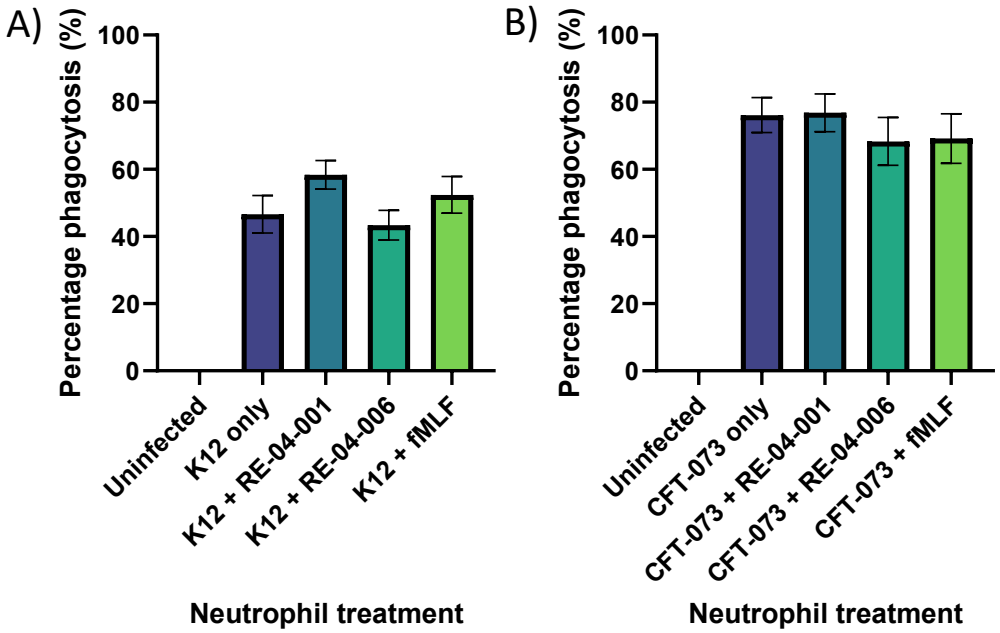

Figure 3.5. The effects of FPR1 stimulation on neutrophil phagocytosis of *E. coli.*

E. coli strains K12 (A) and CFT-073 (B) were co-cultured for 45 minutes with neutrophils; in the presence and absence of FPR1 stimulating compounds. Data shows the percentage of neutrophils that had phagocytosed at least one bacterium (n = 5 independent donor neutrophils).

Error bars represent the mean ± SEM. A one-way ANOVA examining the total number of neutrophils observed and another examining the number of phagocytosed neutrophils concluded that treatment with RE-04-001, RE-04-006 or fMLF had no significant impact on phagocytosis under the parameters of the assay.

3.3.3 Viability of Clinically Relevant Strains

To explore whether time of incubation with neutrophils and FPR1 agonists had any effect on the viability of *E. coli* strains, hourly time points were studied for up to 3 hours (n=1). Only RE-04-006 was used in this work to ensure data could be produced in a short period of time. At 1 hour post incubation (Figure 3.6A) the bacteria only controls (BOCs) of the three clinical strains showed a lower CFU/ml than inocula co-cultured with neutrophils, where a reduction in percentage survival of up to 35% was observed. Contrarily CFT-073 observed a higher CFU/ml in the BOC than the neutrophil co-cultures, though the difference was slight. Strain 84's BOC had the lowest percent survival compared to the initial inoculum at 37.5%, and strain 73 had the highest at 79%. At 2 hours post incubation (Figure 3.6B) the average CFU/ml of co-cultures with strains CFT-073, 73 and 84 was up to 59% greater than that of the BOCs and 63 was down 48%. This trend maintained at 3-hours post incubation (Figure 3.6C) except for strain 84 wherein the BOC CFU/ml rose to match that of the co-culture.

Strain 73's BOC also saw a notable 33% decrease in CFU/ml compared to the previous 2-hour BOC. Addition of RE-04-006 had varying results; at 1 hour post incubation an increase in CFU/ml was observed in treated samples of CFT-073 and 84 when compared to those with neutrophils only, whilst 63 and 73 displayed a reduction. At 2 hours incubation treated samples infected with CFT-073 and strain 73 displayed a lower CFU/ml than untreated samples. The inverse was observed in samples infected with strains 63 and 84. At 3 hours post-incubation this trend persisted except in strain 73 where CFU/ml was higher in the treated sample than the untreated sample.

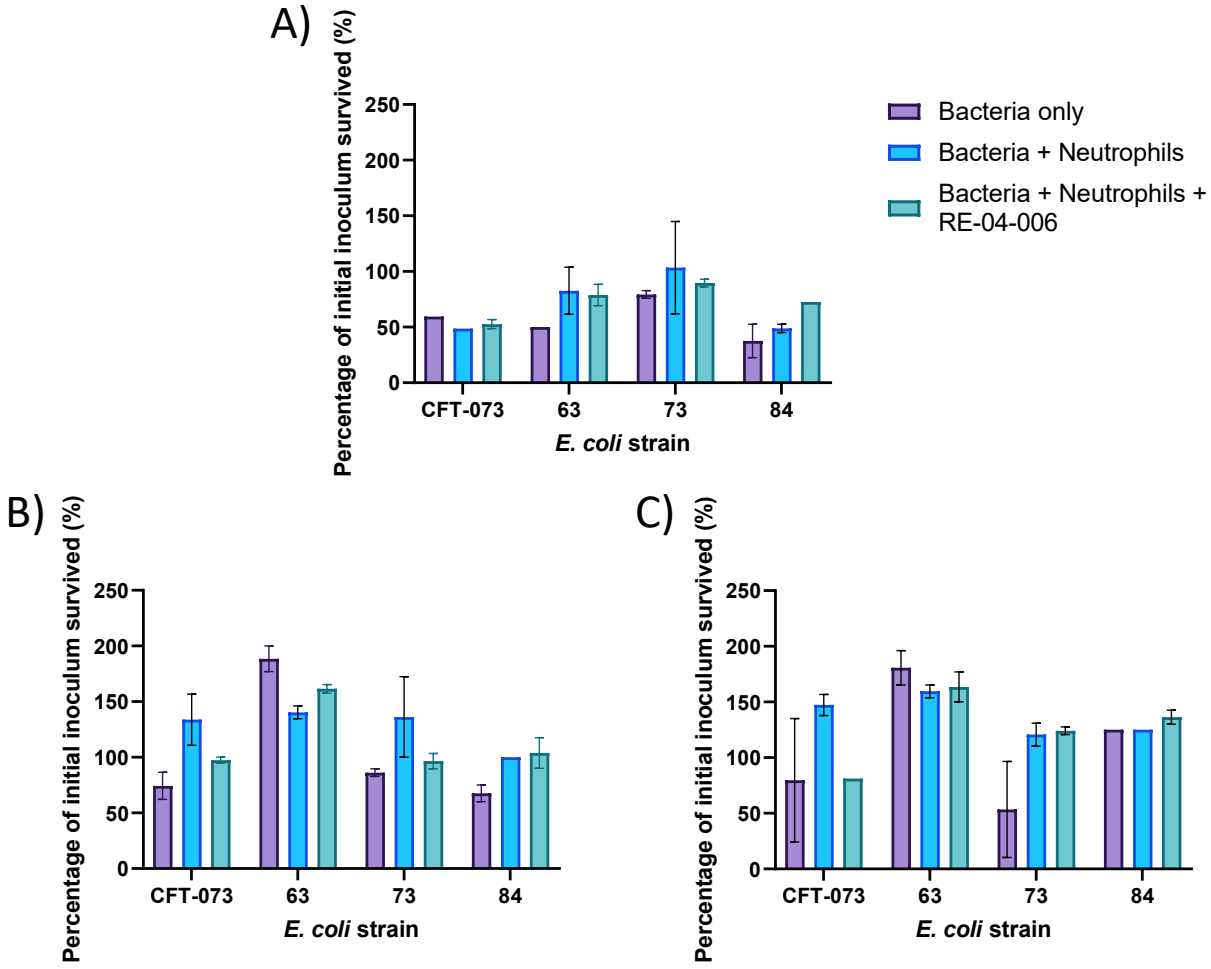

Figure 3.6. The impact of FPR1 stimulated neutrophils on *E. coli* viability.
Neutrophils and *E. coli* were co-cultured in a tube rotator for (A) 1 hour (B) 2 hours or (C) 3 hours, plated on agar and the CFU/ml of each inoculum was calculated following incubation overnight. Data expressed as mean of n=1 experiments. Error bars represent the variance between two technical replicates.

3.4 Flow Cytometry

3.4.1 Neutrophil Characterization

Flow cytometry was employed to analyse the size and granularity of neutrophils following treatment with FPR1 stimulating compounds (Figure 3.7). No notable differences could be seen between untreated and treated samples, and the neutrophil population remained distinct and with no significant changes in size (FSC) or granularity (SSC). Cell death was confirmed in Triton X-100 treated samples (Figure 3.7E). The Triton X-100 control saw a significant reduction in the distinct population of neutrophils and a large increase in the population of cellular debris in the lower quartile of the plot, indicating significant cell death and a breakdown of cellular morphology.

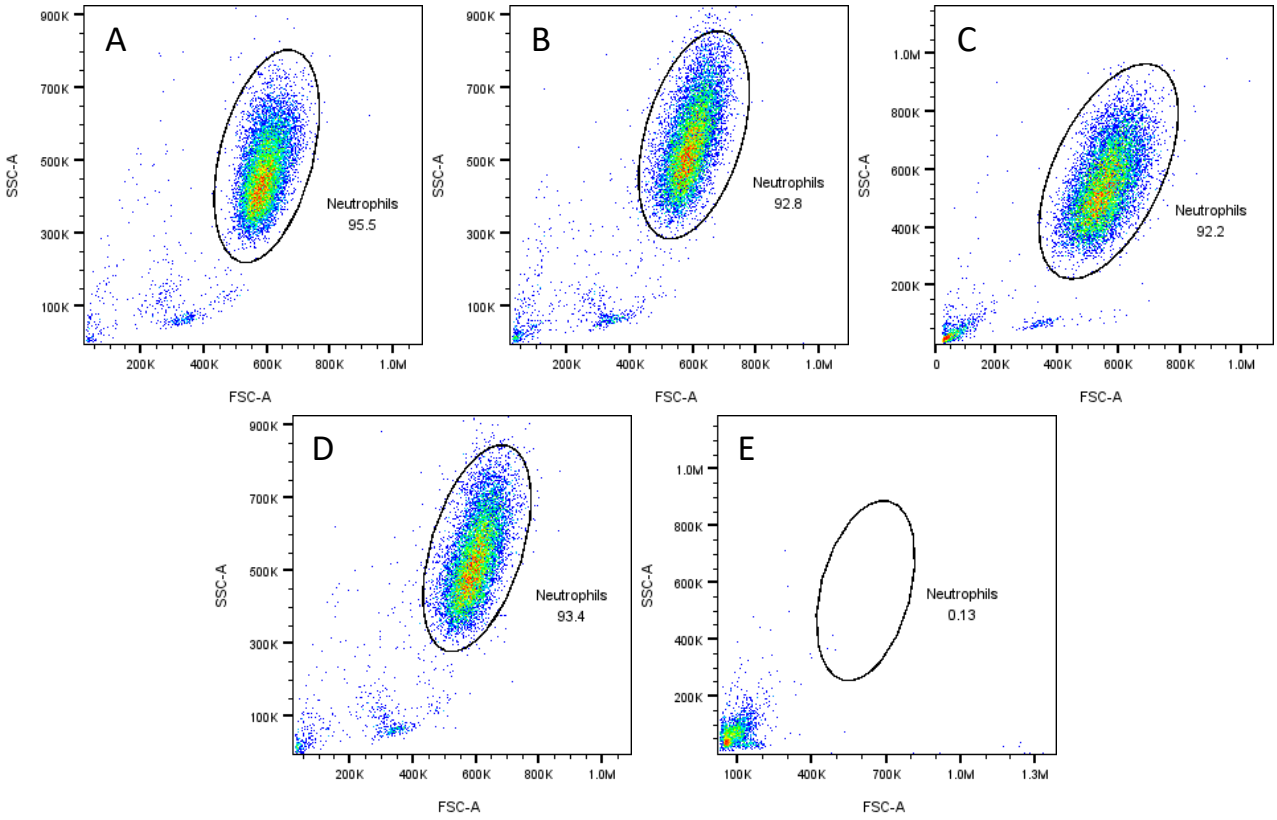

Figure 3.7. The effects of FPR1 stimulating compounds on neutrophil morphology and viability. Neutrophils were isolated from whole blood and incubated for 1 hours (A), select samples were treated with RE-04-001 (B), RE-04-006 (C) or fMLF (D) for 1 hour and then stained with DRAQ7 for 10 minutes. Additionally, a triton control (E) was prepared and gated using a separate sample. Data shows scatter plots of the cells plotted as FSC-A against SSC-A. Samples have undergone doublet discrimination and neutrophils have been gated in each plot.

3.4.2 Neutrophil Viability Assay

A DRAQ7™ neutrophil viability assay was employed to assess the survival of stimulated neutrophils in the presence of compounds and bacteria. No significant differences were observed in percentage death (Figure 3.8A), median fluorescence intensity (Figure 3.8B) or percentage neutrophils within the gate (Figure 3.8C) between any samples (excluding triton X-100). Samples treated with RE-01-006 also trended towards a reduction in percentage neutrophils in the gated population by up to 10%. Triton X-100 controls were observed to have an average percentage death of 82%, an average MFI of 50,245 and an average gated neutrophil percent of 0.62%, all of which were significantly different to every other sample (data not shown).

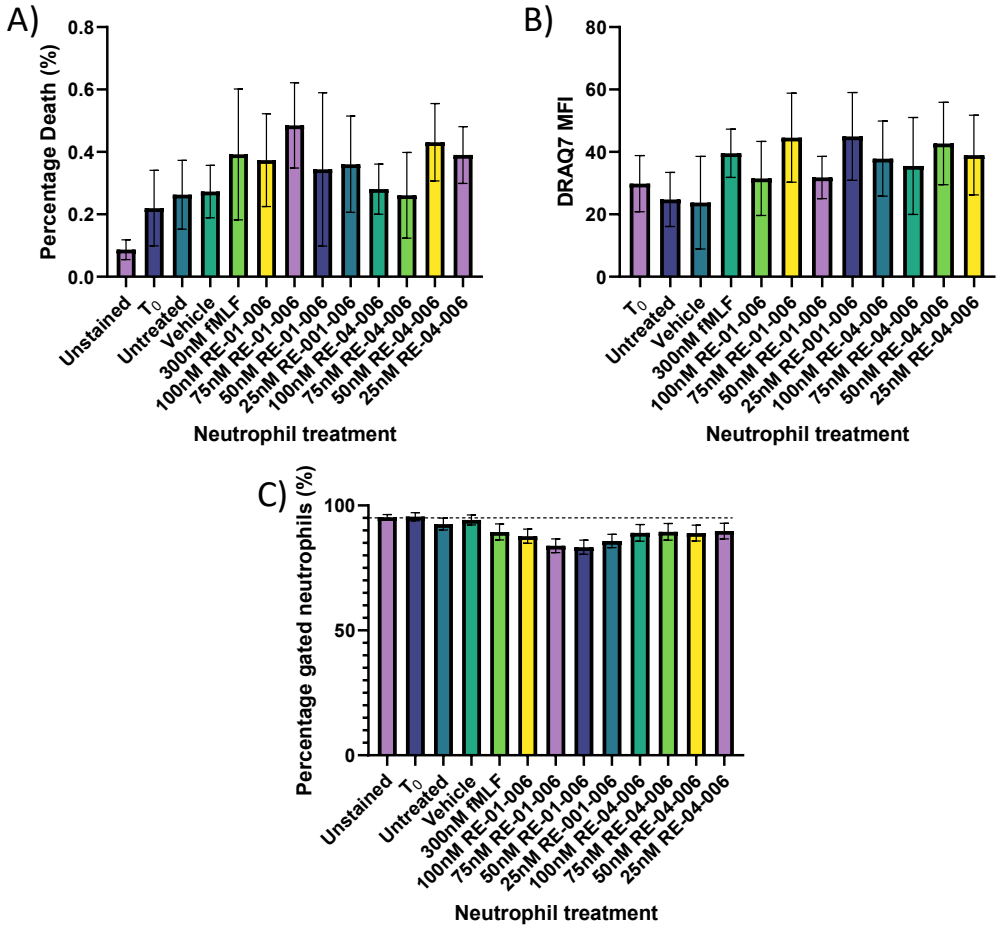

Figure 3.8. Quantitative changes in the viability and morphology of FPR1 stimulated neutrophils. Neutrophils treated with FPR1 stimulating compounds and stained with DRAQ7 were analysed via flow cytometry. (A) A triton control was used to generate a gate for the percentage dead cells and applied to all other samples. (B) The median fluorescence intensity of the DRAQ7 stain in each sample was recorded. (C) A gate identifying viable neutrophils was applied to every sample to record the percentage of viable neutrophils in each population. Data is presented as the mean ± SEM (N = 4 independent donor neutrophils). Data was analysed with a one-way ANOVA and Tukey's multiple comparison test, but no significance was found.

3.4.3 Co-culture Characterization

Neutrophil morphology and viability were also assessed following a 45-minute co-culture with bacteria (Figure 3.9). This was to ascertain if the combined stimulation of both bacteria and compounds was negatively affecting neutrophil viability, potentially interfering with other assays. Neutrophils co-cultured with K12 (Figure 3.9A) and CFT-073 (Figure 3.9B) displayed a less distinctive but more diverse population of neutrophils when compared to uninfected samples (Figure 3.7). Treatment with RE-04-001 (Figure 3.9C, D), RE-04-006 (Figure 3.9E, F) or fMLF (Figure 3.9G, H) further diminished the distinctiveness of the neutrophil population. A notable shift towards the bottom left quartile of the plot (where cells are smaller and less granular) was seen in all infected samples but was most present in FPR1 agonist-treated samples. Finally, there was an increased number of larger (higher FSC) and more granular (higher SSC) cells within each population.

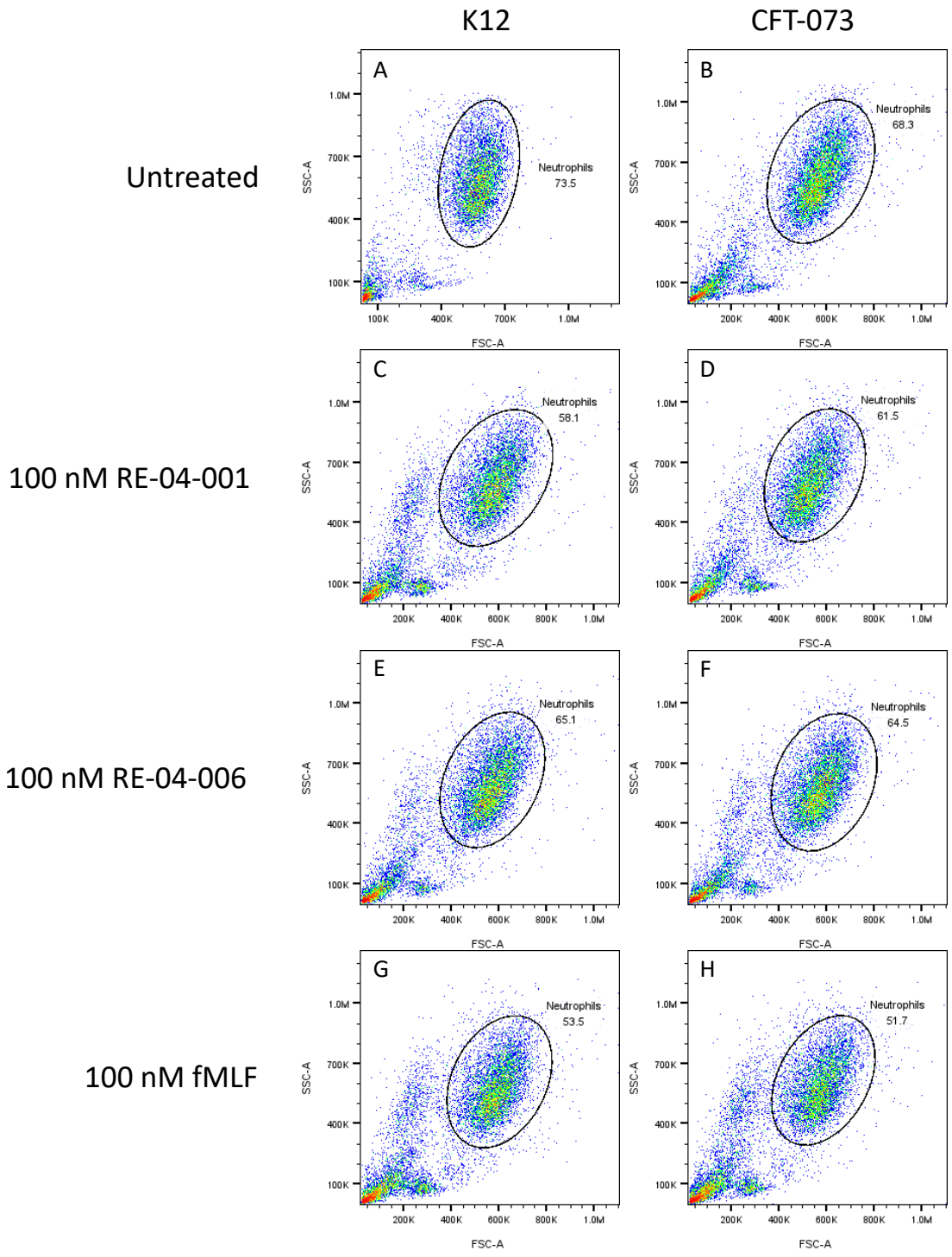

Figure 3.9. The combined effects of *E. coli* infection and FPR1 stimulation on neutrophil viability and morphology.

Neutrophils isolated from whole blood and *E. coli* strains K12 and CFT-073 were co-cultured and treated with FPR1 stimulating compounds for 45 minutes. Samples were stained with DRAQ7 for 10 minutes and then analysed via flow cytometry. Samples underwent doublet discrimination and neutrophils were gated in each plot. Data shows scatter plots of the cells plotted as FSC-A against SSC-A.

3.4.4 Neutrophil Population Analysis

Changes in the appearance of the neutrophil population observed in the scatter plots were quantified into three additional populations (Figure 3.10). Neutrophils co-cultured with K12 or CFT-073 (Figure 3.10A) exhibited a 7-8% increase in the population of smaller cells (Q1 cells) when compared to the uninfected controls (Figure 3.8). Treatment with RE-04-001, RE-04-006 and fMLF showed a 1-2% decrease in this population for K12 co-cultures and a 2-3% decrease for CFT-073 co-cultures when compared to untreated co-cultures.

A significant increase in the smaller and less granular populations (Q3 cells) was observed in neutrophils co-cultured with K12 or CFT-073 when compared to the three uninfected controls (Figure 3.10B). Treatment with fMLF, RE-04-001 and RE-04-006 increased the size of this population by up to 10% when compared to untreated controls with the effect being most noticeable in fMLF treated samples. CFT-073 co-cultures treated with RE-04-006 instead saw a 4% decrease away from this population.

A 3-4% increase in the number of more granular and/or larger cells (Q4 cells) was observed in neutrophils co-cultured with K12 or CFT-073 when compared to the three uninfected controls (Figure 3.10C). Treatment with fMLF, RE-04-001 and RE-04-006 enhanced this population by a small amount (up to 1.2%) when compared to the untreated control except for CFT-073 co-cultures treated with RE-04-006 which saw a minor decrease.

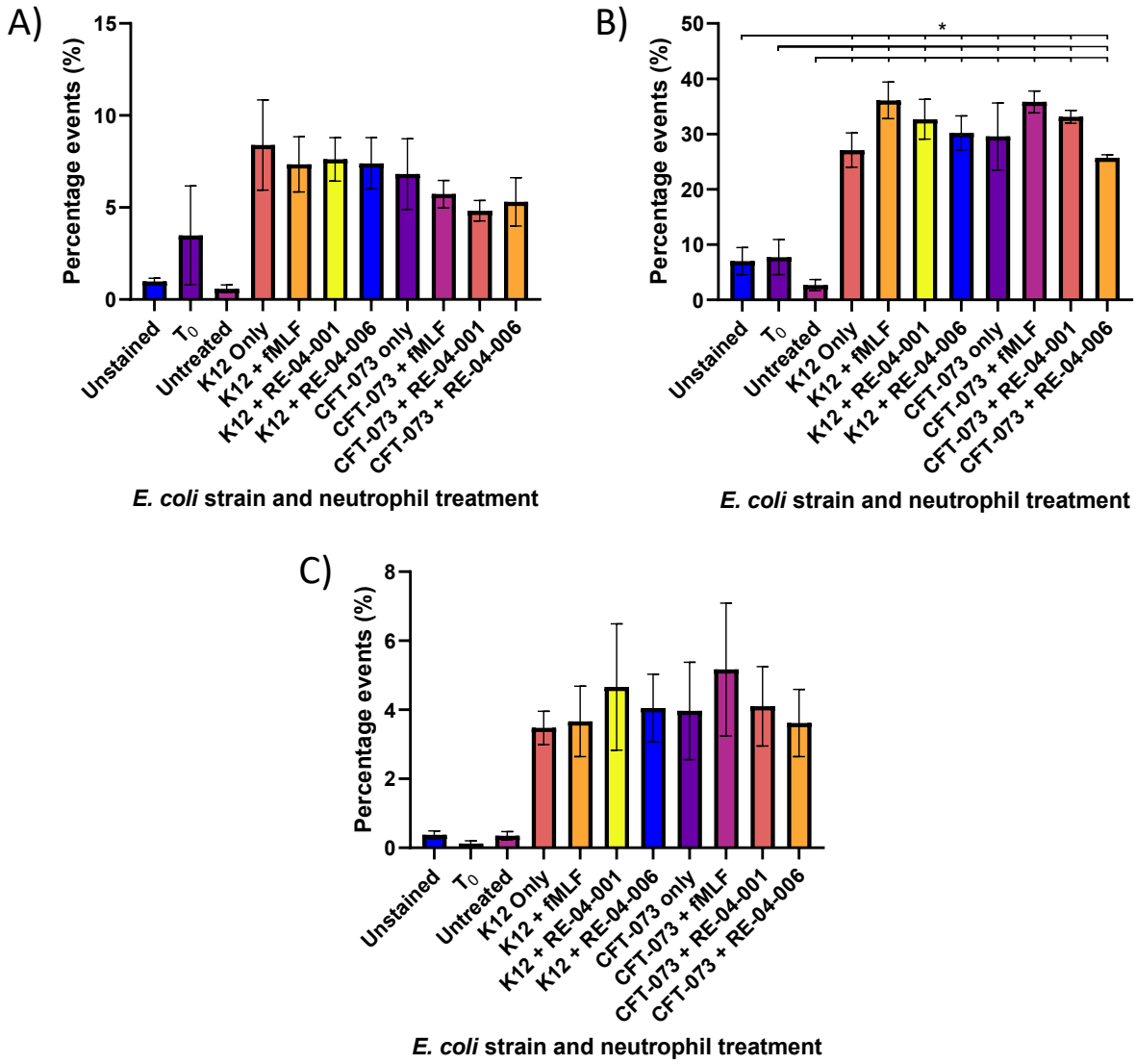

Figure 3.10. Changes in size and granularity in co-cultured neutrophils.

Additional gates were applied to co-cultured populations to identify cells with a lower FSC (A), a lower FSC and SSC (B) or a larger FSC and/or SSC (C) than the gated neutrophil population. Data is presented as the mean +/- SEM (n=4 independent donors). Data was analysed with two one-way Anova's and Tukey's multiple comparison tests, the first on the total number of events detected and the second on the total number of events in the respective gate.

3.4.5 Co-culture Viability Assay

Morphological changes and NET formation was assessed by confocal microscopy. An additional DRAQ7 viability assay was performed on neutrophil bacterial co-cultures to determine the impact of infection on neutrophil viability. No significant differences in percentage death (Figure 3.11A) or MFI (Figure 3.11B) were detected between any samples. However, addition of *E. coli* strains K12 and CFT-073 triggered a significant reduction in the population of gated neutrophils when compared to the uninfected untreated control (Figure

3.11C). Similarly, a significant difference was observed between the T_0 control samples infected with K12, and K12 + RE-04-001. A similar trend was followed with CFT-073. No significant changes in gated neutrophils were detected between untreated infected samples and infected samples treated with RE-04-001, RE-04-006 or fMLF.

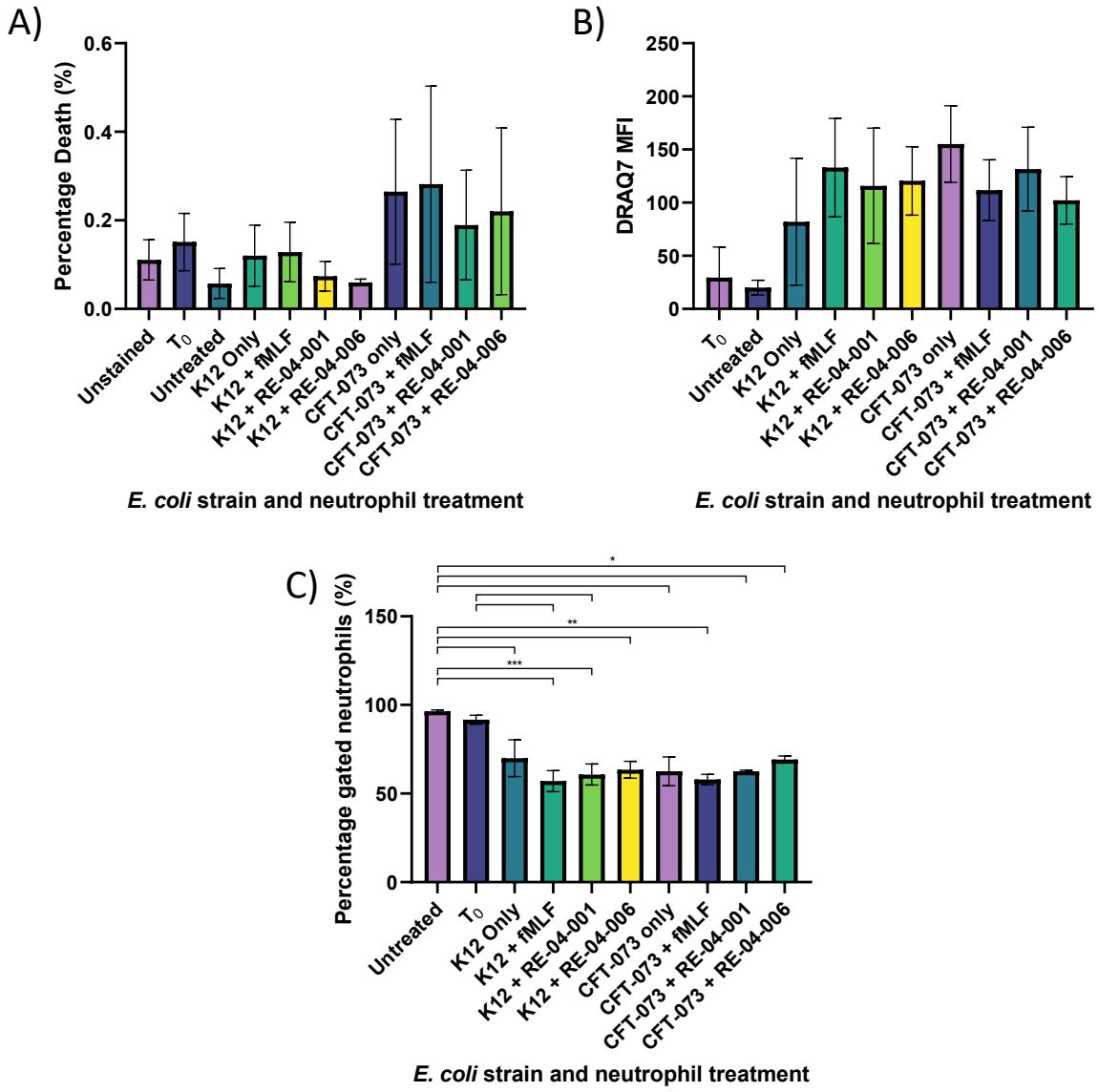

Figure 3.11. Changes in viability and morphology of neutrophil *E. coli* co-cultures.
Co-cultures of neutrophils and *E. coli* strains K12 and CFT-073 were treated with FPR1 stimulating compounds for 45 minutes and stained with DRAQ7. A triton control was used to generate a gate for the percent dead cells which was applied to all other samples (A). The median fluorescence intensity of the DRAQ7 stain in each sample was recorded (B). A gate identifying typical neutrophils was applied to every sample to record the percentage of neutrophils in each population (C). Data is presented as the mean ± SEM (N = 4). Data was analysed with a one-way ANOVA and Tukey's multiple comparison test.

3.5 Confocal Microscopy

Morphological changes and NET formation was assessed by confocal microscopy (Figure 3.12). Treatment with PMA, RE-04-001 and RE-04-006 for 3 hours stimulated the production of NETs in neutrophils (Figure 3.12B, C, D). Addition of *E. coli* K12 (Figure 3.12E) or CFT-073 (Figure 3.12F) did not appear to induce the production of NETs after 1 hour but did alter the morphology of neutrophils. Neutrophils co-cultured with bacteria were smaller and the nuclei appeared condensed, an effect which was more noticeable in CFT-073 infected samples. The Triton X-100 control (Figure 3.12G) demonstrated a complete breakdown of cellular morphology wherein the nuclei became much larger and appeared decondensed.

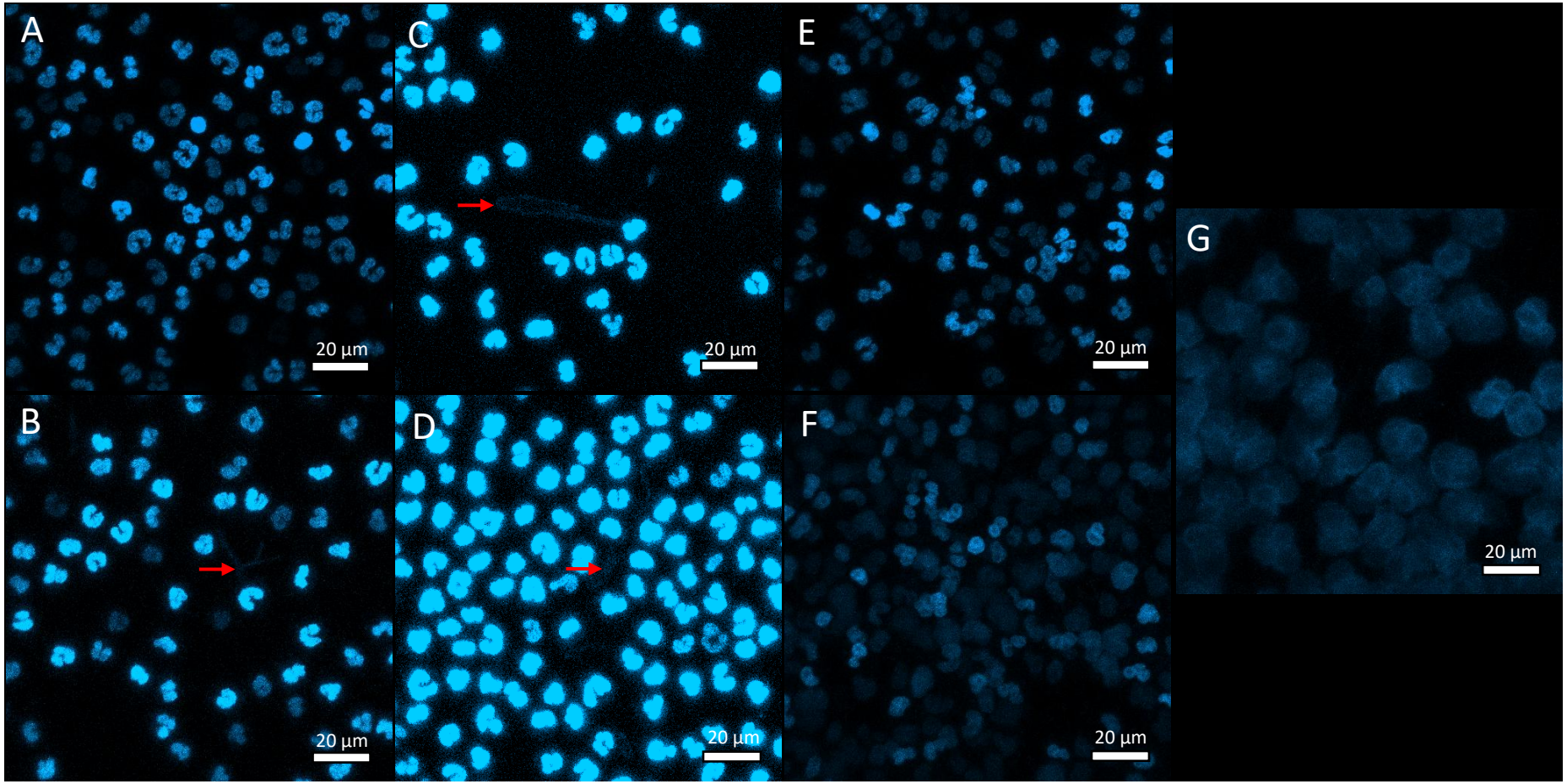

Figure 3.12 The effect of neutrophil stimulation or co-culture with *E. coli* on NETosis.

Z-stacks of neutrophils generated using confocal microscopy. (A) Untreated neutrophils (B) Neutrophils treated with PMA (C) RE-04-001 or (D) RE-04-006 (E) Neutrophils co-cultured with *E. coli* strain K12 or (F) CFT-073 (G) Neutrophils lysed with Triton X-100. The red arrows highlight NETs originating from neutrophils. Images B, C and D have boosted contrast to aid in the visualisation of NETs, for unmodified images see appendix D. Images were obtained using a x63 lens under oil immersion. All samples are stained with DAPI. n=1.

Chapter 4: Discussion

Uropathogenic *E. coli* possesses the ability to resist the bladder's innate immune response and then flourish within the bladder by establishing a niche within bladder cells to evade immune cell action and using oxidative stress resistance systems to endure neutrophil killing. These mechanisms lead to high rates of recurrent infections and frequently require the intervention of antibiotics which contributes to the rising global trends of AMR. Immunotherapies targeting neutrophils in UPEC are a potential alternative to antibiotics, theoretically enhancing the initial innate response to a degree that UPEC are eradicated early, preventing persistence. This thesis aimed to characterise the interactions between FPR1-stimulating compounds, neutrophils and UPEC independently and in co-culture to determine the suitability of these compounds as antimicrobial immunotherapeutic drugs.

4.1 *E. coli* Tolerance to Oxidative Stress

The first aim of this work was to identify *E. coli* strains with varying tolerances to oxidative stress to be used in characterizing the killing responses in FPR1-stimulated neutrophils. Five *E. coli* strains were assessed, two commonly used reference strains, K12 and CFT-073, which have been well-characterized in the literature (Kaper, Nataro and Mobley, 2004), and three UPEC clinical isolates, 63, 73 and 84 (Wilkinson, 2022). UPEC strain CFT-073 demonstrated the highest tolerance to H_2O_2 and non-pathogenic K12 demonstrated the lowest which was expected (Figure 3.1). This trend is consistent with previous findings, such as those by Loughman and Hunstad (2011) who found that CFT-073 and another UPEC strain were more resistant to oxidative killing than three K12 strains after 2 hours of exposure. Being a pathogenic strain, CFT-073 contains more VFs that enhance its survival under stress. This is supported by previous work as Hryckowian and Welch (2013) observed that the oxidative stress system RpoS responds faster and more robustly in CFT-073 compared to K12 granting it a greater tolerance to H_2O_2. Similarly, Mehta *et al* (2015) observed that the CFT-073 genome contained 29 more binding sites for the oxidative response system NsrR than K12. The large spikes in growth observed in K12 and clinical strain 73 at 0.01% H_2O_2 could indicate enhanced growth at low concentrations of H_2O_2. Sublethal exposure to H_2O_2 and other ROS has been observed to enhance the resistance to subsequent challenges in *E. coli* and alter its metabolic profiles, however enhanced growth has not been recorded and thus these differences may simply be due to inoculum variability (Bhat *et al.*, 2015; Rodríguez-Rojas *et al.*, 2020). More repeats would be needed before any conclusions could be made.

4.2 FPR1-stimulated Killing in Neutrophils

Treatment of *E. coli* reference strains K12, CFT-073 and clinical strains 63, 73 and 84 directly with FPR1 stimulating compounds RE-04-001, RE-04-006 and fMLF had no impact on their growth over 24 hours. This was expected as FPR1 is a mammalian receptor and is not expressed in prokaryotic *E. coli*, and thus the compounds cannot interact with bacteria via this receptor (Boulay *et al.*, 1990). These results also imply that RE-04-001, RE-04-006 and fMLF have no additional interactions with UPEC, suggesting that they are specific to FPR1.

This thesis observed a trend towards reduced throughput of UPEC strain CFT-073 in gentamicin killing assays after one hour when stimulated via FPR1 with fMLF, RE-04-001, and most notably RE-04-006. This result could indicate enhanced bacterial killing in stimulated neutrophils, though no statistical significance was observed. While FPR1 is known to play a key role in the activation of the oxidative burst, work investigating enhanced stimulation in the context of infection is sparce. Work in FPR1-deficient mice revealed a significant impairment of the oxidative burst and bacterial clearance in co-cultures with *E. coli* and *Listeria monocytogenes* (Liu *et al.*, 2012; Zhang *et al.*, 2020). Nafiz *et al* (2024) observed significantly enhanced neutrophil killing of *Mycobacterium tuberculosis* in neutrophils treated with FPR1-stimulating fMLF compared to an untreated control after 24 hours. Additionally, this enhanced killing ability was lost in neutrophils lacking oxidative protein $gp91^{phox}$ or PAD4, an enzyme critical in NETosis, suggesting that the oxidative burst and NETosis play a key role in FPR1-driven bacterial clearance. Weiß *et al* (2019) similarly observed that treatment with fMLF enhanced neutrophil killing of *S. aureus* over a 1-hour period. FPR1 stimulation is known to contribute to all forms of neutrophil killing and is important during the oxidative burst (Migeotte *et al.*, 2006). Thus, fMLF, RE-04-001 and RE-04-006 treated neutrophils likely have an enhanced killing ability compared to untreated neutrophils, reflected in a loss of bacterial viability. Furthermore, FPR1 stimulation is also crucial to the priming process, wherein the oxidative machinery is partially assembled allowing for a faster, more efficient and robust response to subsequent stimulation (Daniels *et al.*, 1994). This would result in stimulated neutrophils being able to mount a more extensive inflammatory response towards UPEC than the untreated control.

A greater impact on the throughput of the gentamicin killing assays was observed in neutrophils stimulated with RE-04-001 and RE-04-006 compared to fMLF assuming that UPEC were taken up to an equivalent level as K12 and were killed intracellularly prior to

neutrophil lysis (Figure 3.4). Following stimulation by fMLF and other agonists, neutrophil GPCRs will stimulate various downstream signalling events to induce the oxidative burst, however they will also recruit β-arrestin, a protein involved in receptor desensitization and signal attenuation (Wang and Ye, 2022). Literature suggests that compared to fMLF, the RE compounds only weakly induce the recruitment of β-arrestin potentially allowing for prolonged stimulation and a more extensive oxidative burst which would in turn have stronger bactericidal effects (Lind *et al.*, 2020).

Contrary to expectations, FPR1 stimulation did not enhance the neutrophil killing of K12, instead trending towards enhancing bacterial survival. Furthermore, a greater amount of K12 was seen in lysates from neutrophils compared to CFT-073. This is unusual as K12 is known to have a lower oxidative tolerance than CFT-073 (Figure 3.1) and has been previously demonstrated to be more susceptible to neutrophil killing than UPEC (Loughman and Hunstad, 2011). This difference is further compounded by the phagocytosis data (Figure 3.5) which indicated that up to 33% more CFT-073 was phagocytosed than K12. More phagocytosis would allow more bacteria to be protected from gentamicin treatment within neutrophils, theoretically enhancing the throughput of the assay and yet more K12 was observed following incubation, again possibly suggesting a higher tolerance to neutrophil killing. Conversely it is possible that neutrophil survival following the uptake of CFT-073 is impaired compared to K12, resulting in a perceived reduction of bacteria following the gentamicin assay. Under normal physiological conditions this would be normal as the dead neutrophil and its contents would be phagocytosed by other phagocytes, namely macrophages, however in the context of this assay it may have impacted the amount of bacteria outputted by the assay (Cox, Crossley and Xing, 1995).

A possible source of this unusual behaviour is the K12 strain itself. The strain provided by the MID lab has been used in research by many researchers for a long period of time and has likely diverged significantly from the strain first isolated in 1922 and the strains used by other researchers. During this time the strain may have developed mutations which enhance its resistance to methods of neutrophil killing, it may also have acquired resistance to gentamicin, allowing extracellular *E. coli* to ‘contaminate’ the final results. This is unlikely however as the concentration of gentamicin used was well above the recorded MIC of K12 and even CFT-073 (EUCAST, 2024).

It is also possible that as a commensal strain, K12 stimulates weaker antimicrobial activity from neutrophils compared to the pathogenic CFT-073. In mast cells, pathogenic *E. coli* have been demonstrated to elicit a much greater granular response than non-pathogenic commensals (Malaviya *et al.*, 1994; Magerl *et al.*, 2008; Krämer *et al.*, 2008). This has yet to be investigated in neutrophils but could partially explain K12's higher counts within surviving neutrophils.

Bacterial survival within the neutrophil may have impacted the trends seen in this assay. Nazareth *et al* (2007) observed that compared to other ExPEC and UPEC strains, many of which had low tolerance to oxidative stress, CFT-073 displayed poor survivability within neutrophils to the point that they were unable to successfully retrieve any samples following a killing assay. Fexby *et al* (2007) were able to retrieve K12 following a similar killing assay for a similar time period, suggesting that K12 may survive the neutrophil environment better than CFT-073. This would in turn impact the 'throughput' of the assay, diminishing CFT-073 survival following incubation.

The bacterial viability data collected in this work suggests that the presence of neutrophils negatively impacts bacterial viability after 1 hour of incubation, but that the bacteria are able to overcome this challenge after 2-3 hours (Figure 3.6). However, this considers both intra- and extracellular bacteria and crucially due to time constraints, this experiment was unable to be repeated, so the role of *E. coli* viability on perceived *E. coli* killing has not yet been elucidated.

It is also important to consider that the perceived differences in survivability may be due to methodology. Figures 3.1 and 3.2 show that CFT-073 replicates faster and to a greater extent than K12 in LB broth over 24 hours. This trend may not be the same in the neutrophil complete media or on the LB agar.
Finally, as discussed, the gentamicin protection assay suffered from very high intra-subject variability and low statistical power. Further repeats are needed to determine if any significant impact on UPEC or K12 is derived from FPR1 stimulation in neutrophils.

4.3 Extracellular Killing of *E. coli* by Neutrophils

This thesis observed that treatment with FPR1 stimulating compounds enhanced the extracellular killing of K12 but hindered the extracellular killing of CFT-073 (Figure 3.4). The role of extracellular ROS in bactericidal activity is hotly debated. FPR1 expression on

the neutrophil surface is limited and ROS may be unable to accumulate to toxic concentrations outside the confinement of the phagosome (Ulfig and Leichart, 2021). Some research has hypothesized that outside the cell ROS only serves in signalling roles and in the initiation of other killing methods such as degranulation, NETosis and HOCl generation (Roos, Bruggen and Meischl, 2003).

Importantly, the absence of DRAQ7 fluorescence (Figure 3.8) suggests that NETosis does not occur after an hour. Thus, the extracellular killing assay, where a 1-hour incubation is used may exclude one of the most significant extracellular mechanisms available to neutrophils, limiting its accuracy to provide a genuine neutrophil response in UPEC. Clearly, further repeats with longer incubations could result in an enhanced killing of *E. coli*. It is also important to consider that CFT-073 replicates much more rapidly than K12 (Figure 3.1), and unlike in the intracellular compartment where replication is inhibited, both strains would be rapidly multiplying outside the neutrophil (Helaine *et al*., 2010)

Much like the intracellular killing assay, high donor neutrophil variability was observed as reflected by the large error bars for each treatment (Figure 3.4). This limitation should be put in context considering the primary cellular material used in each experiment, increasing the relevancy of the work. Nevertheless, more repeats are necessary before any final conclusions can be made on the role of FPR1 stimulation in extracellular killing.

4.4 Impact of FPR1 on Phagocytosis of *E. coli*

This work observed that stimulation of FPR1 induced no significant changes in the phagocytosis of *E. coli* CFT-073 and K12 (Figure 3.5). FPR1 is known to have a role in the stimulation of phagocytosis in neutrophils, however literature suggests that it is not essential to the process. Work utilising FPR1-deficient neutrophils has demonstrated that the absence of FPR1 has no significant impact on the phagocytosis of pathogenic motifs (Liu *et al.*, 2012; Leslie *et al.*, 2020). It is possible that instead FPR1 enhances the phagocytic process by working in tandem with phagocytic complement and Fcγ receptors. Contrary to this, Weiß *et al* (2019) observed a significant increase in phagocytosis of a bacteraemia *E. coli* strain, as well as *S. aureus, S. epidermidis,* and *S. lugdunensis* and *Listeria monocytogenes* in neutrophils stimulated with 500 nM fMLF compared to an untreated control. However, Weiß *et al* utilised heat inactivated bacteria compared to the live bacteria used in this work. Heat inactivated bacteria are less readily phagocytosed than live bacteria as the heat inactivation process denatures immunogenic proteins on their surface, hindering neutrophil recognition (DeChatelet *et al.*, 1974). Thus, neutrophils may be aided by enhanced FPR1 stimulation.

Weiß *et al* also used neutrophils isolated with a density gradient. Interestingly, Blanter *et al* (2022) observed that neutrophils isolated via negative selection with magnetic beads, the method used in this work, phagocytosed *S. aureus* coated beads more readily than neutrophils isolated with a density gradient. They also observed that magnetically separated neutrophils expressed higher levels of FPR1 and higher levels of ROS production, NETosis and degranulation when stimulated with *E. coli* LPS. It is therefore possible that in this work the more phagocytically active neutrophils interacted with the more immunogenic live bacteria to such a degree that FPR1 'assistance' was inconsequential.

4.5 Impact of FPR1 Stimulation on Neutrophil Morphology and Viability

Neutrophil morphology and viability were investigated using flow cytometry and DRAQ7. As a potential therapeutic it is important that FPR1 stimulation has no negative impact on neutrophil viability as this could hinder their effectiveness and harm a patient. No changes in neutrophil viability were observed following 1 hour of FPR1 stimulation. This is consistent with the current literature, as Pajonczyk *et al* (2024) observed no impact on the survival of neutrophils treated with fMLF over 20 hours and found that in in neutrophils stimulated with LPS, fMLF treatment actually significantly enhanced survival over a 20-hour incubation.

Similarly, stimulation of FPR1 in neutrophils co-cultured with *E. coli* K12 and CFT-073 had no significant impact on viability, nor did the presence of *E. coli* in general. The presence of pathogens is known to have varying effects on neutrophil viability. Detection and interaction with pathogens enhances neutrophil survival by delaying apoptosis so that neutrophils can exert a more robust inflammatory response (Kobayashi, Malachowa and DeLeo, 2017). However, bacterial toxins and neutrophil-derived ROS can induce neutrophil death at high enough concentrations (Watson *et al.*, 1996; Kobayashi *et al.*, 2002). In this work neutrophils were only exposed to bacteria for 1 hour, and it is likely that changes in viability would be seen in more prolonged co-cultures. Engelich, White and Hartshorn (2001) observed that co-culture with *Streptococcus pneumoniae* reduced the number of viable neutrophils in suspension by 40% over 24 hours *in vitro*, however the decline in viability began at 4-5 hours post inoculation. Similarly, Pleskova *et al* (2024) observed a significant increase in neutrophil apoptosis over a four-hour incubation when treated with pathogenic *E. coli*.

Neutrophils co-cultured with *E. coli* demonstrated a distinct shift in morphology towards a smaller and less granular phenotype, which appeared to occur independently of FPR1 stimulation (Figure 3.10). The decrease in neutrophil granularity (reflected as a decrease in

SSC) is easy to rationalise, following stimulation neutrophil granules will mobilise to the phagosome and cell surface to expel their contents. Granule proteins are detectable outside the cell within seconds to minutes of neutrophil stimulation and so following 1 hour of stimulation the neutrophils will likely have expelled a large portion of their granular arsenal, which itself won't replenish for several hours (Pryzwansky *et al.*, 1979; Sengeløv *et al.*, 1995; Nauclér *et al.*, 2002). This decrease in granularity was not observed in neutrophil-only samples even when treated with FPR1 stimulating compounds. Mol *et al* (2021) observed that two individual stimuli are required for efficient activation and degranulation of neutrophils which would be provided in the presence of bacteria and FPR1 stimulating compounds, but not with the compounds in isolation.

FPR1 stimulation resulted in a significant increase in the detection of smaller events, especially in the lower left corner of the intensity plot, typically indicating a greater concentration of cellular debris. Literature suggests that neutrophils will become smaller during the late stages of NETosis after the nuclear material has been expelled and during apoptosis (Bortner and Cidlowski, 1998; Kobayaski, Malachowi and DeLeo *et al.*, 2017), however both these processes as well as any processes that would generate cellular debris would have been reflected by a decrease in neutrophil viability. It is feasible that stained extracellular DNA from apoptotic or NETosing neutrophils was lost during the washing process and therefore absent from the final reading. The triton control was excluded from the washing procedure as it was found to severely impact the number of detectable events. However, co-cultured neutrophils generated a suitable number of events even after washing. Unfortunately, the flow cytometer could not be loaded with unwashed samples, so this could not be tested. To verify that the method is functioning as intended, future work could test the discarded supernatant from the wash for DRAQ7™ stained DNA using fluorescence microscopy or using a dsDNA assay.

Yang *et al* (2019b) observed a very similar shift in morphology in neutrophils co-cultured with *S. aureus* using flow cytometry and recorded a considerable drop in viability using an alternative DNA marker. However, their work co-cultured neutrophils for two hours longer than this work, has been observed to have an impact on viability.

4.6 FPR1-driven NETosis

NET-like projections were observed in neutrophils treated with PMA, RE-04-001 and RE-04-006 (Figure 3.12). PMA has previously been observed to strongly induce NETosis and was

expected to follow this trend (Hoppenbrouwers *et al.*, 2017). The presence of NET-like structure in RE-04-001 and 006 treated neutrophils suggests that FPR1 stimulation induces NETosis. Indeed, FPR1 stimulated with fMLF is known to induce NETosis in neutrophils (Pruchniak and Demkov, 2019). However, the stain used in the neutrophil viability assay, DRAQ7™, binds to extracellular DNA including projected NETs and lysed cells killed during NETosis, yet no increase in fluorescence was observed in stimulated neutrophils after one hour (Figure 3.8). This is unusual as DRAQ7™ is known to be sensitive to NETosis and has been used as a marker for the process in other research (Biermann *et al.*, 2016; Janko *et al.*, 2023).

This is likely due to a disparity in incubation times. To ensure that the PMA positive control sufficiently stimulated NETosis, cells were treated for 3 hours, whereas neutrophils in the viability assay were only treated for an hour. Allen *et al* (2022) observed a significant increase in NETosis between neutrophils co-cultured for 20 minutes and 2 hours, similarly Pruchiak and Demkov (2019) only observed minor NETosis after 1 hour of fMLF treatment, with a notable increase after 3 hours.

NETosis was not observed in neutrophils co-cultured with *E. coli* K12 or CFT-073. This contradicts findings in the literature where *E. coli* have been observed to strongly induce NETosis after 3-4 hours (Marin-Esteban *et al*., 2012; Chi and Sun, 2016). Phagocytosis of large particles such as apoptotic cells has been demonstrated to inhibit NETosis, however it is unlikely that the small *E. coli* would be able to inhibit NETosis (Manfredi *et al.*, 2018).

The NET projections were very difficult to identify within the samples and almost were invisible in unmodified images. This was thought to be due to insufficient DAPI staining, thus it is possible that NETs produced by co-cultured neutrophils may simply not be visible under conditions used in this work. Future work including more concentrated DAPI and potentially stains for NET proteins such as elastase would better elucidate the interactions between FPR1, *E. coli* and NETosis. Additionally, following cell fixing samples underwent a wash with PBS. It is possible that the delicate NET structures were washed away or disrupted during this step leaving them absent from the final results. Future work could test the supernatant for DNA with DNA stains such as DRAQ7 to ensure that the wash step has no impact on the viability of FPR1 stimulated NET structures.

Limitations and Future Work

Various limitations and potential avenues for future work were highlighted throughout the thesis.

Early experiments with isolated neutrophils faced issues with the cells being too 'sticky', tending to adhere to each other and the plate, requiring significant manual disruption to resuspend. It was important that this disruption was also not too 'rough' as that could alter the viability and activity of the neutrophils, thus it took several initial repeats before this process was optimised (Ekpenyong *et al.*, 2015). This very likely impacted the throughput of early experiments, potentially resulting in the loss of neutrophils and bacteria, in turn impacting the reliability of earlier replicates.

In the majority of experiments outlined in this thesis (excluding the NETosis assay), neutrophils were stimulated with FPR1 agonists and co-cultured with bacteria for 45-60 minutes. This was initially due to issues with neutrophil viability in incubations longer than one hour, and while this was eventually optimised, the one-hour time-point was maintained for consistency**.** While this single timepoint was suitable for the base characterisation of interactions between FPR1, neutrophils and UPEC, it only provides a limited window into the dynamics of these interactions. A typical UTI can last days to weeks and during this time the immune and infectious environment within the bladder can change substantially (Abraham and Miao, 2015). Future work could include incubations at 6 hours to represent the early influx of neutrophils into the site of infection, or potentially work up to 24 hours, though this might lead to issues with neutrophil viability due to their short half-lives and the lack of immunomodulation from other cells (Abraham and Miao, 2015; Lahoz-Beneytez *et al*., 2016). Additionally, it is also important to ensure that FPR1 treatment has no adverse effects on neutrophils over a longer period of time, and to observe the impact of treated neutrophils on bacteria for incubations longer than an hour.

Temporal changes have clear physiological relevance. As time progresses in a UTI, neutrophils will eventually be downregulated by signals from other immune cells (Adrover *et al.*, 2020). FPR1 stimulating compounds may have the potential to offset this downregulation, thus maintaining a bactericidal environment within the bladder beyond what is usually physiologically capable. The literature suggests that bacteria can have a significant impact on neutrophil viability over longer periods of time. FPR1 stimulation may alter this dynamic, perhaps clearing enough bacteria so that neutrophils remain viable for longer, or perhaps 'over-stimulating' neutrophils and inducing apoptosis earlier.

A similarly pertinent direction for future research is shorter time intervals, perhaps taking measurements every 10-20 minutes. This would provide improve detail into how FPR1 stimulation shapes the initial bacterial challenge in neutrophils, the impact of FPR1 priming and better elucidate the role of FPR1 stimulation on UPEC overall. This would also enable the calculation of rates as opposed to the simpler metrics of percentage phagocytosis or CFU/ml used in this work.

In the early stages of this project there were plans to characterise the impact of FPR1 stimulated neutrophils on the viability of SV-HUC-1 BECs, an immortalised human urothelial cell line. SV-HUC-1 cells are non-tumorigenic and when cultured form a urothelial monolayer that serves as a stable phenotypical model of the urothelial barrier *in vitro* (Reznikoff et al, 1988). As a potential therapeutic it is critical to determine the toxicity profile of RE compounds in cells that mimic the environment of intended use. Oxidative stress is also known to alter bladder function which would need to be considered (Masuda *et al.*, 2008). Viability was to be investigated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which assesses mitochondrial function and serves as a good target for assessing oxidative damage as the function of the mitochondria is sensitive to oxidative stress (Bhatti *et al.*, 2017). Work was also planned to investigate the impact of FPR1 stimulated neutrophils on UPEC invasion of the bladder cells using the gentamicin protection assay. This work was discontinued by the failure of a CO_2 incubator before any significant data could be collected and thus serves as an excellent starting point for any future investigations.

One clear strength of this work was the use of isolated neutrophils, which were chosen to identify the interactions between FPR1 and neutrophil activity without the interference of other immune factors. However, a crucial avenue of future research is characterising the impact that FPR1 stimulated neutrophils have on other components of the immune system and ultimately how this impacts bacterial clearance. A whole blood model would provide better insight into the impact FPR1 stimulation has on the innate immune response to UPEC as other immune cells would be present to support neutrophil function and alter the inflammatory response (Chick *et al*., 2024). It would also aid in understanding the impact of FPR1 stimulation on other immune cells, especially via enhanced ROS production. However, a whole blood model would poorly reflect the cellular dynamics during a UTI. Following infection there is an early influx of neutrophils which will be supported by resident mast cells, macrophages and BEC signalling (Agace *et al.*, 1995**)**. Whole blood is rich in many

types of immune cells, platelets and immune proteins which would substantially alter the nature of the immune response to UPEC. An organoid model might better reflect the cellular dynamic in the bladder, several of which have been developed in recent years (Vasyutin *et al.*, 2019; Sharma *et al.*, 2021; Flores *et al.*, 2023).

FPR1 is quite a promiscuous receptor and is not limited to expression on neutrophils. FPR1 can be found at varying levels on all innate leukocytes, dendritic cells, adaptive T and B cells, platelets, various epithelial cells and even lightly expressed on glial cells and astrocytes in the central nervous system (Migeotte *et al*., 2006). Further research into the interactions between FPR1 stimulating compounds and these other cells would further elucidate their therapeutic potential. Work by another researcher at Swansea University revealed that treatment with RE-04-001 and 006 enhances the activation of $CD4^+$ T-cells as well as neutrophils (Data unpublished).

Minimal statistical significance was seen throughout this work. This may be due to the high biological variability between donors in isolated neutrophils. Performing repeat experiments with the same donor was difficult as donors were only able to donate once every 2 months and often donors were not able to donate on a regular basis. Power calculations on this data project a high number of subjects necessary to avoid subject variability.

Considerable donor to donor variability was observed in the data, a power calculation aiming for a statistical power of 80% and a significance level of 0.05 determined that 76 donors would have needed to be studied to mitigate subject variability when analysing viable counts from the cell lysate. The same calculation on the phagocytosis data similarly revealed that 65 subjects would be needed. These values represent a significant amount of time and coordination but also a good avenue for future work investigating the RE compounds. Additionally, efforts could be made to eliminate variability. Data from the gentamicin assay generally demonstrated the same trends between donors but absolute values were very far apart, logarithmic transformation of the data may help in minimising the impact of donor variability on the data.

Blood collection was certainly a limitation of this work. The process of finding and organising donors was difficult, and blood was typically only available 1-2 times a week. Additionally, as neutrophils are so short-lived blood had to be used on the same day it was collected. This limited the amount of neutrophil work that could be done in a week and therefore contributed to the lack of power in our studies. Despite these restrictions this work

optimised assays for use with primary cells from human subjects and generated data that informed future power calculations.

Conclusions

Identifying possible alternative or synergistic therapies to antibiotics is vitally important in the ongoing battle against AMR in the urinary tract and beyond. Due to their critical role in UTIs, neutrophils are a promising target for immunotherapy in the bladder. This work highlights that targeted FPR1 stimulation via small molecule compounds in neutrophils enhances NETosis and the oxidative burst, which in turn results in superior clearance of UPEC *in vitro*. Importantly, this work also demonstrated that extraneous FPR1 stimulation does not impact the viability of neutrophils under the given conditions. This is crucial if they are to be considered for potential therapeutic use as any drug-derived damage to the immune system could actively worsen a UTI. The RE compounds conceptually show promise as a therapeutic agent as they clearly demonstrate an ability to enhance neutrophil killing of UPEC, however the degree of killing shown in this work is non-significant. Greater killing could be achieved with a higher dosage or perhaps by co-stimulation of another receptor. However, enhancing the oxidative burst to such a degree that it could reliably eradicate a UTI would likely cause significant damage to the urinary tract. Instead, it is more plausible that FPR1 stimulating compounds could be used in conjunction with conventional antibiotics, providing a boost to the immunity and hopefully completely eradicating UPEC. This could aid in preventing the development of chronic UTIs and slowing the development of AMR.

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Appendix A. Gene Selection Table

Table 4.1 Genes assessed in clinical UPEC isolates

Oxidative stress factor	Gene	Function	Reference
Sigma factor RpoS	*rpoS*	Sigma factor directs gene expression in response to ROS stress. *E.coli* lacking rpoS show defects in colonisation. Potentially also involved in downregulating fimA during stationary phase	Hryckowian and Welch, 2013
Periplasmic protein yqhG	*yqhG*	Enhances expression of type 1 fimbriae and grants resistance to hydrogen peroxide, mechanism unknown.	Bessaiah *et al*., 2019
Small RNA RyfA	*ryfA*	Grants resistance to oxidative stress, alters expression of genes involved in stress responses, metabolism and biofilm formation	Bessaiah *et al*., 2021
Invasion protein IbeA	*IbeA*	Confers H_2O_2 resistance	Flechard *et al*., 2012
Alkyl hydroperoxide reductase	*ahpC, ahpF*	Breaks down H_2O_2 and hydroperoxides into water and alcohol, additionally primary scavenger for H_2O_2 at lower concentrations	Welch *et al*., 2002
Peroxiredoxin Bcp	*bcp*	Thiol peroxidase that catalyses the reduction of H_2O_2 and various hydroperoxides.	Welch *et al*., 2002
Methionine sulfoxide reductase	*msrA, msrB*	Repairs proteins that have been inactivated by oxidation	Hochhut *et al*., 2006
Superoxide response regulon	*SoxS, SoxR*	Sense increasing concentrations of superoxide and induce ~25 genes including superoxide dismutases, endonucleases and dehydrogenases to minimize damage	Mehta *et al*., 2015
Polyphosphate kinas 1-coding gene	*ppk1*	Enhances oxidative stress resistance, modulates expression of katG and katE	Ching-Ye *et al*., 2017
Catalase E/G	*KatE/G*	Monofunctional catalase HPII, breaks down H_2O_2 into water	Vigil, Alteri and Mobley, 2011
Superoxide dismutases	*sodA, sodB, sodC*	Use iron and manganese to break down superoxide into oxygen and H2O2	Fee, 1991
Oxidative resistance and hemolysis kinase/regulator	*orhK, orhR*	Activate putative methionine sulfoxide reductase system and hemolysin in response to H_2O_2, enhancing resistance. Also thought to trigger host cell pyroptosis	Gu *et al.*, 2021

Oxidative stress Regulator OxyR	*oxyR, oxyS*	Induces the transcription of ~30 antioxidant genes which reduce H_2O_2 concentration, sequester iron and repair oxidative damage	Chiang and Schellhorn, 2012
Glutathione peroxidase	*btuE*	Decomposes hydrogen peroxide with glutathione	Arenas *et al*., 2011
Thiol peroxidase	*tpx*	Primary antioxidant during anaerobic growth	Kim *et al*., 1996
Glutathione reductase	*gor*	Enzymatically restores glutathione	Welch *et al*., 2002

Appendix B. Disc diffusion data

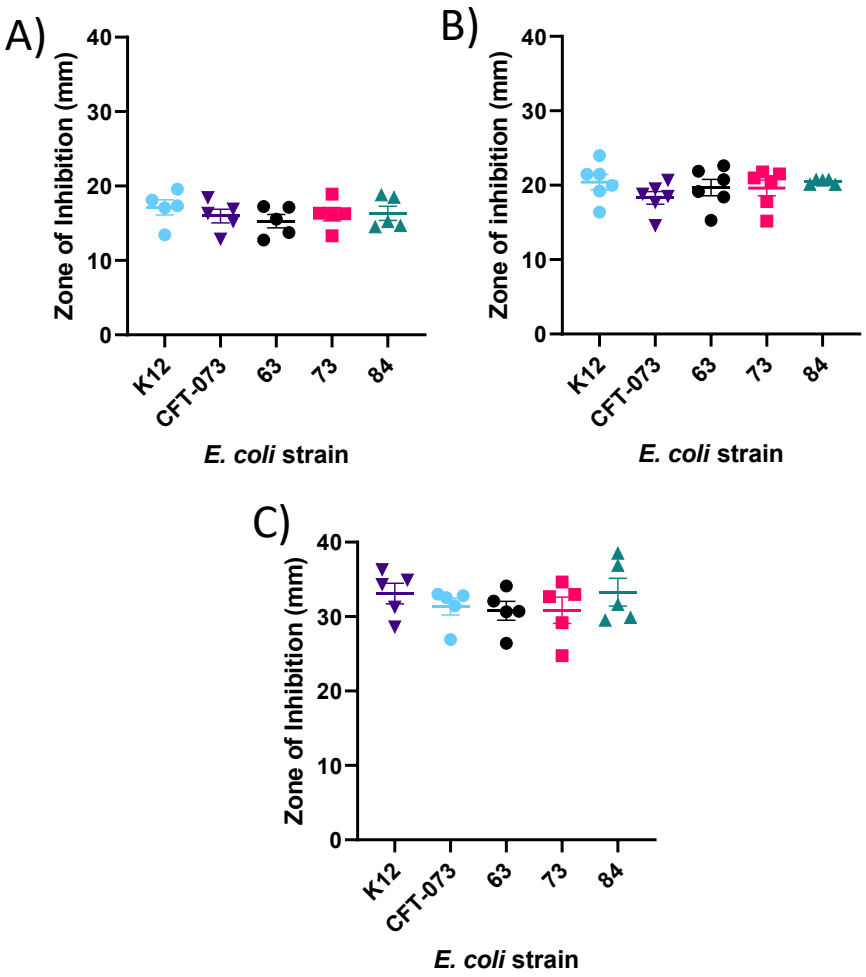

Figure 4.1 The effect of H_2O_2 on *E. coli* growth.

Paper discs infused with H_2O_2 at concentrations of 1% (A), 3% (B) and 30% (C) were pressed into agar plates that had been streaked with five different strains of *E. coli* and incubated overnight at 37 C. Plots display the zones of inhibition in mm around each disk ± SEM (n=3). A one-way ANOVA determined no significance between strains ($P<0.05$).

Appendix C. Microscopy images

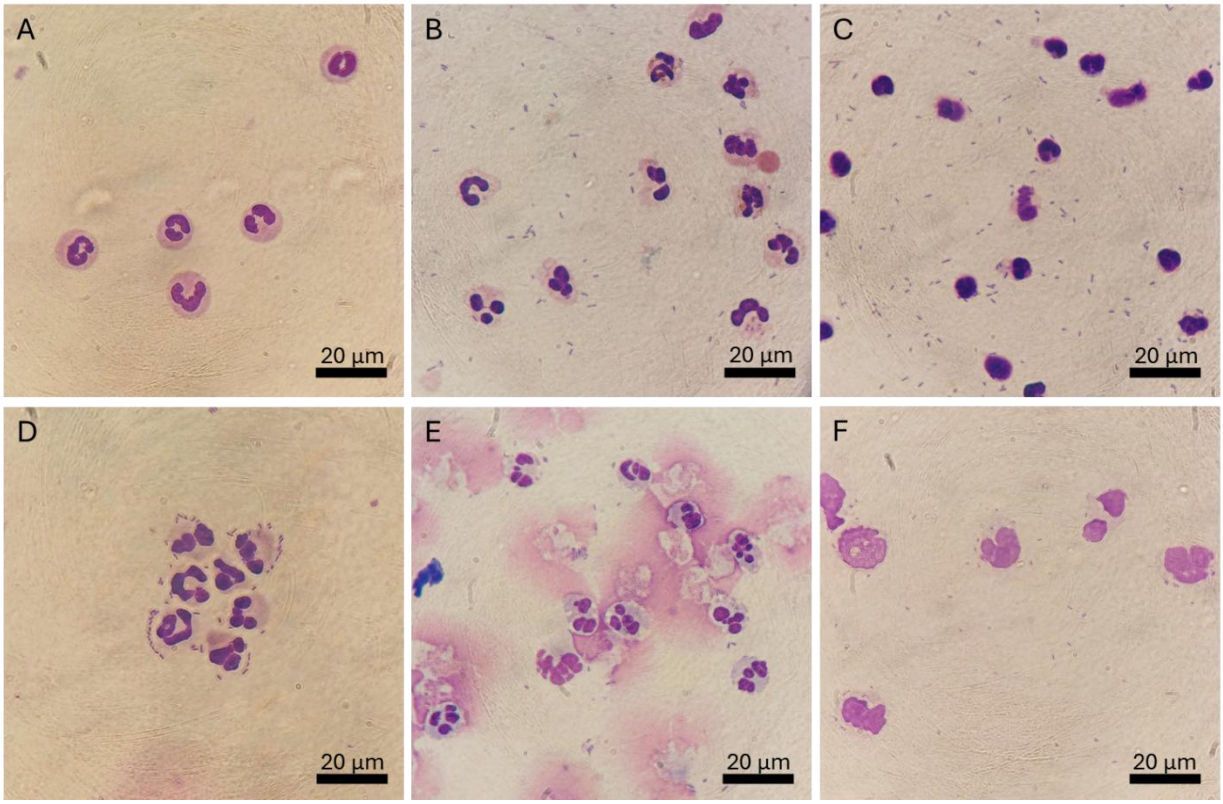

Figure 4.2 Neutrophil morphology following *E. coli* co-culture

Neutrophils displayed a wide range of morphologies when analysed with microscopy, these changes appeared independently of FPR1 stimulation but only appeared in *E. coli* co-cultures. A: Untreated uninfected neutrophils. B: Infected neutrophils with normal morphology. C: Infected neutrophils with decondensed nuclei. D: Infected neutrophils clustering together. E: Infected neutrophils amidst lysed neutrophils. E: Fully decondensed and swollen infected neutrophils.

Appendix D. Unmodified NET images