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Activation of the inflammasome in bovine mammary macrophages by *Streptococcus uberis*

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Abstract

Bovine mastitis is an inflammatory disease of the mammary gland of cattle that negatively impacts animal welfare, economics and productivity within the dairy industry. *Streptococcus uberis* is the most prevalent intramammary pathogen in the UK and is becoming increasingly important globally. In contrast to other mastitis pathogens, *S. uberis* does not stimulate an innate immune response from epithelial tissues. Initial host recognition of infection is via macrophages found within milk, resulting in a delayed response and subsequent damage to the mammary gland leading to clinical signs of infection. The interaction between *S. uberis* and bovine mammary macrophages dictates the outcome of infection. Challenge studies with *S. uberis* in dairy cattle have indicated the importance of a bacterial cell surface serine protease, SUB1154, which is essential for colonisation leading to disease. Therefore, the aim of this project was to characterise the interaction between SUB1154 and the host macrophage with respect to detailing its role in disease pathogenesis.

A highly reproducible and reliable method for isolating bovine mammary macrophages from milk was developed. A hallmark for immune activation in macrophages is the production of the pro-inflammatory cytokine IL-1β via the NLRP3 inflammasomal pathway. Challenge of bovine mammary macrophages with heat-killed strains of *S. uberis* and/or purified SUB1154 demonstrated that in the absence of SUB1154, IL-1β was not produced. Production of IL-1β was restored following supplementation of the SUB1154 deletion mutant strain with the purified recombinant version of the SUB1154 protein. Therefore, a following objective of this study was to determine the role of the SUB1154 protein in the induction of the inflammasomal pathway.

The presence of SUB1154 (but not its putative protease active site domain) was shown to be essential for the production of IL-1β by providing the NLRP3 inflammasome priming signal, inducing pro-IL-1β transcription. Priming of the inflammasome by SUB1154 was blocked by the TLR2 TIR ligand C29 (but not by the TLR2 extracellular binding ligand MMG 11) and by blocking entry of the protein into the cell with cytochalasin D. The requirements to both penetrate the cell and TLR-TIR dependency led to the hypothesis that SUB1154 most likely primes the inflammasome pathway through interactions with bovine TIR domains associated with TLR2.

SUB1154 also appears to play a positive role in *S. uberis* intracellular survival/proliferation within bovine mammary macrophages. This role may contribute towards the reduced

virulence of the SUB1154 deletion mutant. This aspect requires further investigation to confirm and extend these findings. Additionally, macrophages challenged with different *S. uberis* strains induced varying concentrations of IL-1β. However, no evidence was found to support the greater/lesser production of IL-1β being related to sequence variation within SUB1154 or the amount of SUB1154 expressed by the *S. uberis* strains. The extent of IL-1β production could be speculated to be dependent on the NLRP3 inflammasome activation signal(s), which requires further elucidation. Understanding the bovine immune response to *S. uberis* will help in the discovery of potential stages of pathogenesis against which immunomodulatory therapeutics may be developed.

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Supplementary information

List of abbreviations

0140JΔ*sub1154* - *S. uberis* 0140J SUB1154 deletion mutant

ACTB - actin beta

AIM2 – absent in melanoma 2

ANOVA – analysis of variance

ASC - apoptosis-associated speck-like protein containing a caspase recruitment domain

BD – binding domain

BHI – brain heart infusion

bMECs – bovine mammary epithelial cells

BMMO – bovine mammary macrophage

bp – base pairs

BSA – bovine serum albumin

BTK - Bruton's tyrosine kinase

CARD - caspase activation and recruitment domain

CASP1 - pro-caspase-1

CCL20 - C-C motif chemokine ligand 20

CD – cluster of differentiation

CDS – coding sequences

CFU – colony forming units

CMT – California mastitis test

CyD - Cytochalasin D

DCT – dry cow therapy

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

ER – endoplasmic reticulum

FACS - fluorescence-activated cell sorting

FBS - foetal bovine serum

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GAS - Group A *Streptococcus*

Gfi1 - growth factor independence 1

GRE1 - Gli-responsive element 1

GWAS - genome-wide association studies

HEK – human embryonic kidney

HRP - horseradish peroxidase

IFI16 – interferon inducible protein 16

IFN – interferon

Ig – immunoglobulin

IKK complex - IκB kinase

IL – interleukin

ILC3 – type 3 innate lymphoid cells

IMDM - Iscove's modified Dulbecco's medium

IMI – intramammary infection

IP_3R - inositol triphosphate receptor

IPTG - isopropyl β-D-1-thiogalactopyranoside

IRAK - interleukin-1 receptor associated kinase

ISS1 – insertional element

iTOL – interactive tree of life

IκB - inhibitor of nuclear factor-κB

LB - Luria-Bertani

Lbp – lactoferrin binding protein

LF – lactoferrin

LPS – lipopolysaccharide

LRR – leucine rich repeat

LTA - lipoteichoic acid

MAC-T cells – bovine mammary epithelial cell line

MAFFT - multiple alignment using fast fourier transform

MAL - MyD88-adaptor like

M-CSF - macrophage colony-stimulating factor

MEGA 11 - molecular evolutionary genetics analysis 11

MGS - modified Gey's balanced salt solution

MHC - major histocompatibility complex

MiST – microbial signal transduction

MOI – multiplicity of infection

MS – mass spectrometry

MyD88 - myeloid differentiation 88

NACHT - NLRP3 nucleotide-binding and oligomerisation

NADPH - nicotinamide adenine dinucleotide phosphate hydrogen

NCBI – national centre for biotechnology information

NEK7 - never in mitosis gene A (NIMA)-related kinase 7

NETs – neutrophil extracellular traps

NF-κB - nuclear factor kappa B

NK cells – natural killer cells

NLR – NOD-like receptor

NLRC4 - NLR family CARD domain containing 4

NLRP3 - nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3

NOD - nucleotide-binding oligomerisation domain

NT – no treatment

OD – optical density

PAE – PBS, acid-citrate, EDTA

PAMPs – pathogen associated molecular patterns

PBMCs - peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE-Cy7 – phycoerythrin cyanine 7

PIP_2 - phosphatidylinositol 4,5-bisphosphate

PRRs – pattern-recognition receptors

PVDF - polyvinylidene fluoride

PYD – pyrin domain

RAW 246.7 – murine macrophage cell line

RLR - RIG-1 like receptors

ROS – reactive oxygen species

RPL13a - ribosomal protein 13

rSUB1154 – recombinant SUB1154 protein

rSUB1154NP – proteolytically compromised recombinant SUB1154 protein

RT qRT-PCR - Real-Time quantitative Reverse Transcription Polymerase Chain Reaction

SARM - sterile α- and armadillo-motif-containing protein

SCC – somatic cell count

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SIFT - sorting intolerant from tolerant

SrtA – sortase A

STING – stimulator of interferon genes

T3SS – type 3 secretion system

TAB - TAK1 binding protein

TAK1 - transforming growth factor-β-activated kinase

TBST – tris buffered saline with Tween

Tcps - TIR domain containing-proteins

TCR – T-cell receptor

TE – tris EDTA

TF – transferrin

Tfh – T-follicular helper cell

Th – T helper

THB – Todd-Hewitt broth

TIR – toll-interleukin receptor

TIRAP - TIR domain-containing adaptor protein

TLR – toll-like receptor

TNF-α – tumour necrosis factor alpha

TRAF6 - TNF receptor-associated factor 6

TRAM - TRIF-related adaptor molecule

Treg – T-regulatory cell

TRIF - TIR domain-containing adaptor protein inducing IFN-β

TTP2 - tripeptidyl-peptidase 2

Chapter 1: Introduction

1.1. Bovine mastitis

Bovine mastitis is an inflammatory disease of cattle that negatively impacts animal welfare, economics and productivity within the dairy industry (Watts, 1988; Klass & Zadoks, 2018). It is most often a result of bacterial infection of the mammary gland, with more than 135 bacterial species identified as causative agents (Watts, 1988; Klass & Zadoks, 2018).

1.1.1. Signs of disease

Clinical infections present as localised inflamed udder quarters which are enlarged, hard, hot and painful upon palpation, accompanied by reduced milk production and an abnormal milk secretion often containing aggregate flakes and/or clots (Jones & Bailey, 2009; Blowey & Weaver, 2011; Klass & Zadoks, 2018). In some cases, systemic signs, such as an elevated temperature, may also be present (Laven, 2016).

Although intramammary infection can lead to obvious and overt signs of mastitis, subclinical infection can also occur with these signs absent. Despite milk having a normal appearance, more detailed analysis often reveals changes to its biochemical and/or cellular composition. In regard to cellular composition, intramammary infection is often accompanied by a higher-than-normal somatic cell count (SCC) due to the influx of neutrophils into the mammary gland (Jones & Bailey, 2009; Blowey & Weaver, 2011; Klass & Zadoks, 2018). The pathologies observed largely result from the host response to the infection.

1.1.2. Causes of bovine mastitis

Intramammary bacterial infection is considered to be the main cause of bovine mastitis. The bacterial species responsible for the majority of mastitis cases worldwide are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* (Krishnamoorthy *et al*., 2021). Bacterial intramammary infection is classified based on the bacterial origin, contagious or environmental. Contagious mastitis occurs through transmission between cows, most commonly during milking. Maintenance of milking equipment and post-milking teat disinfection can prevent the spread of contagious infections (Smith & Hogan, 1993; Schreiner & Ruegg, 2002; Sharma *et al*., 2011). Environmental mastitis occurs through infection of the udder quarter by pathogens present within bedding, pasture and/or other external environments. Consequently, environmental pathogens are typically

opportunistic and genetically diverse. Control of environmental infections can be achieved by providing a clean housing environment and reducing exposure of teat ends to the pathogen (Bradley, 2002). However, adequate control of infection from the environment has proven difficult to achieve. Consequently, infection with environmental pathogens predominates even in well maintained dairy herds.

1.1.3. Susceptibility to bovine mastitis

Host factors contribute to the susceptibility to mastitis. Pure breed or cross breed high-yielding cattle such as Holstein-Friesian cattle are considered more genetically susceptible to mastitis (Shaheen *et al*., 2016). Udder structure can also contribute to mastitis susceptibility. Cattle with pendular-shaped udders are at a higher risk of intramammary infection (Waller *et al*., 2014). Frequent milking has, over time, been shown to cause wider teat canals that can be permanently partially open, rendering older cattle potentially more susceptible to infections (Kibebew, 2017). During lactation, there is a higher demand for energy, trace elements and vitamins. A lack of which can result in immunosuppression, increasing susceptibility to infection. Therefore, it is essential for proper management of diet during lactations with the appropriate supplements provided (Matsui, 2012; Chandra *et al*., 2013; Bayril *et al*., 2015; Shaheen *et al*., 2016; Kibebew, 2017).

1.1.4. Impact of mastitis on the dairy industry

A major consequence of mastitis is the underproduction of milk. In the UK alone ~1 million tonnes of milk/year is lost due to mastitis and subsequent inflammatory tissue damage in response to infection (AHDB-Dairy, June 2016). Underproduction of milk due to mastitis contributes significantly to the cost of mastitis to the dairy industry ($1.7-2 billion in the US and £200 million in the UK annually with a total impact worldwide of US$35 billion attributed to economic loss (Jones & Bailey, 2009; Tassi *et al*., 2013; Keane, 2019)). This underproduction of milk can be attributed to a reduction in lactation period by ~57 days per case of clinical mastitis (Khan & Khan, 2006). In addition, mastitis also impacts the environmental sustainability of the dairy industry, for example inefficiencies due to mastitis in the UK alone have been estimated to result in an additional 1 million tonnes CO_2e/year due to the extra cattle required to produce the UK's milk supply (UK-VARSS, 2019).

Intramammary infections also pose an animal welfare issue as the disease causes pain (Blowey & Edmondson, 2010). Mastitis is the most common reason for the use of antimicrobials in dairy cows with approximately 3-4 tonnes used in the UK per annum to prevent and control disease (Pyörälä, 2009; UK-VARSS, 2019). Due to the variation in bacteria causing mastitis, broad

spectrum antibiotics have been used over time, with milk from cows on antibiotic therapy still being fed to calves. This could result in pressure for an increase in antimicrobial resistances within the microflora of the gut or the environment, however, there has only been a limited increase in antimicrobial resistance reported in mastitis pathogens. Of the highest priority critically important antibiotics, *E. coli* from neonatal calves displayed a 4.7% resistance to cefotaxime and a 2.4% resistance to enrofloxacin. Tetracycline resistance was the only recognised resistance observed in *S. dysgalactiae*, with 14.3% of *E. coli* and 51% of *S. uberis* isolates showing tetracycline resistance (UK-VARSS, 2021). Despite this, it is important to understand how bacterial species cause intramammary infection to enable improved rational, targeted interventions.

1.1.5. Prevention of bovine mastitis

Pre-1960s mastitis was a major problem within the dairy industry, partly because this was prior to routine use of antimicrobials for infection control. This led to investigations into how bacterial pathogens cause intramammary infections. The predominant pathogens found in 1967 were *S. aureus*, followed by *S. dysgalactiae*, with *S. agalactiae* having the lowest infection rate (Wilson & Kingwill, 1975; Bramley & Dodd, 1984).

To reduce the incidence of mastitis, a five-point control plan was implemented in the late 1960s/early 1970s by the National Institute for Research in Dairying (Kingwill *et al*., 1970; NIRD/MAFF, 1970). The plan focused on improving hygiene during the milking process by the introduction of post-milking teat disinfection and the routine maintenance of milking machines, identification and treatment of clinical cases using routine therapeutic and prophylactic antimicrobial administration and the culling of persistently infected cattle (Kingwill *et al*., 1970; NIRD/MAFF, 1970; Alnakip *et al*., 2014; UK- VARSS, 2014 & 2018; Breen, 2019; Keane, 2019). Hillerton *et al*., 1995, compared historical and contemporary data to evaluate the effect of the control plan on mastitis. This revealed that the control plan had eliminated 73% of clinical cases of mastitis. Due to control of pathogens with a predominantly contagious route of transmission, the relative frequencies of the pathogens causing mastitis had altered with *E. coli* and *S. uberis* becoming the major causes (Bradley, 2002). Ongoing application of the five-point plan (now accepted as good practice in routine dairy husbandry) has maintained this disease aetiology. Despite technological development within the dairy industry, in countries where the five-point plan is still commonly used, *E. coli* and *S. uberis* remain the predominant causes of bovine mastitis (27% and 32% respectively in the UK) (Bradley *et al*., 2007; UK-VARSS, 2019).

In combination with the five-point plan, several other methods have been developed to help prevent mastitis infection. DCT (dry cow therapy) is the administration of prophylactic antibiotics into the udder of non-lactating cattle and is a major use of antibiotics in the dairy industry. Antibiotics remain within the mammary gland at high concentrations for 20-70 days (depending on antibiotics used) killing bacteria present (AHDB; Biggs, 2017; Ricci *et al*., 2017). Internal teat sealants have been used in place of DCT. This method forms a physical barrier in the teat canal preventing bacteria entering the mammary gland and was found to significantly decrease *S. uberis* infections during the non-lactating period (Parker *et al*., 2007; AHDB).

Mastitis has been a long-term issue for the dairy industry that has not substantially changed since the introduction and widespread application of the five-point plan. Despite there being a reduction in intramammary infection caused by *S. aureus* and *S. dysgalactiae* after controlling routine hygiene/dairy cow management, mastitis due to *S. uberis* and *E. coli* persists.

1.1.6. Diagnosis of intramammary infection and mastitis

Despite efforts to prevent mastitis, it is still a common infection and therefore suitable diagnosis practices need to be in place. Clinical mastitis can be diagnosed through examination of the udder; however, this relies on the presence of signs of infection. To prevent severity of infection by reducing host damage and spread between cattle within the herd, other tests are more suitable to detect intramammary infection before signs of mastitis appear.

Measurements of SCC in milk is a convenient surrogate measure of infection. An increase in SCC suggests an increase in cellular influx into the mammary gland in response to infection (Souza *et al*., 2016). Somaticell® and the DeLaval cell counter utilise the SCC and are portable, allowing for convenient usage on milk from individual quarters, cows, or the bulk tank (Rodrigues *et al*., 2009; Godden *et al*., 2017).

The California Mastitis Test (CMT) is one of the most effective techniques in identifying subclinical mastitis during milking. Milk is collected into a paddle with four-compartments and a reagent is added for approximately 30 seconds. This forms a precipitate on the paddle; a distinct gel formation indicates a strong positive mastitis infection, whereas a slight precipitate on the paddle that disappears following paddle movement represents a negative result (Mira *et al*., 2013).

Newer approaches have also been reported in use on larger farms. For example, magnetic nanoparticles were used for the identification of *S. aureus* (Ryskaliyeva *et al*., 2018). Growing bacterial cultures from milk samples remains the most used method for detecting

intramammary infections in diagnostic centres. However, molecular-based techniques, through the evaluation of bacterial taxonomy and phylogeny, have been found to be more sensitive and rapid (Adkins & Middleton, 2018). Whole genome sequencing has improved present screening techniques by determining the pathogen and detecting specific genes such as *mecC* (homolog to the methicillin resistance *mecA* gene) (Paterson *et al*., 2014; Váradi *et al*., 2017). Detection of *mecC* and *mecA* are important as infections with bacteria expressing these genes are resistant to β-lactam antibiotics (Rainard *et al*., 2018a).

1.1.7. Antibiotic therapy as a treatment for bovine mastitis

Antibiotic therapy is the main treatment method for mastitis (Cheng & Han, 2020), with 60-70% of all antimicrobials administered to cattle used for the prevention and treatment of mastitis in Belgium (Stevens *et al*., 2016). Antibiotics, such as penicillin, ampicillin, tetracycline and gentamycin can be administered by intramammary infusion, intramuscular or intravenous injections (Hossain *et al*., 2017). Currently, penicillin G is the main antimicrobial for treating streptococci causing mastitis with resistance yet to be reported (Pyörälä, 2009; UK-VARSS, 2019). For *E. coli* based infections, aminoglycosides, fluoroquinolones and cephalosporins are the antimicrobials routinely used. Cows treated with antibiotics in combination with non-steroidal anti-inflammatory drugs were shown to have lower SCC, better cure rates and increased fertility compared to antibiotics alone (Laven, 2016).

Despite antibiotics being an effective treatment for mastitis, there are negatives to this therapy option. Antibiotic usage contributes to the underproduction of milk. This is because milk collected during antibiotic treatment is discarded as it contains antibiotic residues which cannot be consumed due to the risk of allergies and drug resistances. This also has an impact on dairy fermentation processes as antibiotics in milk kill or reduce the growth of bacteria required for the manufacturer of yoghurts and cheese (Gomes & Henriques, 2016). Antibiotic resistance is also an area for concern in regard to mastitis treatment. Although antibiotics can eliminate intramammary infections, by the time these are administered the mammary gland has undergone an inflammatory reaction that is likely to have caused tissue damage that may decrease subsequent milk production (Zhao & Lacasse, 2008).

1.1.8. Vaccination against bovine mastitis

As the use of antibiotics impacts sustainability of the dairy industry and the combination of internal teat sealants with DCT is only partially preventative, the need to reduce the incidence of new infections and clinical cases relies on the development of efficacious vaccines (Bradley *et al*., 2010). Most of the research conducted on mastitis vaccine development focused on

targeting *S. aureus*, *S. agalactiae* and *E. coli* (Deb *et al*., 2013; Ismail, 2017). ENVIRACOR J-5®, J-VAC® and ENDOVAC-Dairy® are commercially available vaccines against *E. coli* causing mastitis (Kour *et al*., 2023). These vaccines are used to prevent mastitis through administration during and after pregnancy. ENVIROCOR J-5 was found to shorten the duration of mastitis by 41% compared to unvaccinated cows (Zoetis). In a study in which J-VAC was administered to the whole herd, the incidence of clinical mastitis was reduced by 81% (Boehringer Ingelheim). Although the precise mechanisms of protection from J5 vaccines remain unknown, field studies suggest that the reduced severity of disease occurs due to the actions on the systemic immune response rather than local mammary defences. The systemic response would protect the udder due to the anatomical/physiological blood-udder barrier (Erskine, 2012).

Startvac® (Europe and Canada) and Lysigin® (USA) are commercially available vaccines against *S. aureus* causing mastitis. Administration of Lysigin can begin as early as 6 months of age, followed by recurrent booster doses every 6 months (Kour *et al*., 2023). In addition to *S.* aureus, Startvac also targets *E. coli* and is administered to healthy cows during and after pregnancy in herds prone to mastitis infections (EMA). Reported effectiveness of Startvac was found to vary between field studies. A field study investigating the efficiency of Startvac found no decrease in incidence rates of clinical mastitis, but the severity of disease was significantly reduced (Bradley *et al*., 2015). Conversely, another field study observed moderate reduction in incidence rates of new staphylococcal intramammary infections (Schukken *et al*., 2014). In contrast, Startvac was found to be ineffective in improving udder health, milk production and survival (Landin *et al*., 2015). The Startvac trial conducted by Freick *et al*., 2016, concluded that the vaccine was not appropriate for managing *S. aureus* intramammary infections. Efficacious discrepancies were also observed in field studies evaluating the Lysigin vaccine. In some cases, the vaccine was found to decrease severity of disease and reduce the incidence of intramammary infections (Middleton, 2008). However, in other cases, there was no reduction in prevalence or incidence of *S. aureus* intramammary infection (Middleton *et al*., 2009).

UBAC® (Hipra) is the only commercially available vaccine against *S. uberis* intramammary infections. The vaccine displayed some level of efficacy by significantly reducing clinical signs and milk yield losses. However, UBAC was shown ineffective at preventing intramammary infection as all quarters challenged developed clinical mastitis (Collado *et al*., 2018).

Despite decades of research and the progression in vaccine development against bovine mastitis, due to the controversy in data regarding efficaciousness of current mastitis vaccines on the market, these are all deemed unsatisfactory (Rainard *et al*., 2021). A variety of factors

have been identified hindering mastitis vaccine effectiveness, such as the multi-etiological nature of mastitis, the inhibitory effects of milk components on phagocyte function and the expansive area within the mammary gland that requires immune surveillance (Colditz & Watson, 1985; Rainard *et al*., 2022a). Together, these highlight the importance in further understanding the immune response to invading bacterial pathogens to produce a successful therapy.

1.2. Host-pathogen interactions in cattle during intramammary infection

1.2.1. Bacterial invasion of the mammary gland

It has been suggested that the most common route of intramammary infection is from environmental reservoirs; most likely seeded from commensal populations. Bacteria such as *E. coli* and *S. uberis* act commensally within cattle and are commonly found in the gastrointestinal tract (Bradley *et al*., 2007; Klaas & Zadoks, 2018; Keane, 2019). Potential reservoirs of bacteria in the environment include bedding; faeces; heavy (cattle) traffic areas (e.g., in proximity to water troughs); pasture; soil and grass. Although there is no direct evidence linking faecal isolates as the source of intramammary infection, the most likely hypothesis is that *S. uberis* contaminates the environment through faeces as a gut commensal and is maintained within the cattle population by faecal-oral circulation (Zadoks *et al*., 2005; Lopez-Benavides *et al*., 2007). Occasionally, bacteria gain access via the teat into the mammary gland where they act as pathogens. This route of transmission leads to bacteria evading control by the five-point plan of routine hygiene/dairy cow management (Ward *et al*., 2009; Klaas & Zadoks, 2018).

The bovine teat contains several histological and anatomical structures to prevent bacterial access to the mammary gland (Fig 1.1). The teat canal contains a smooth muscle sphincter that acts as a physical barrier to infection by closing the teat canal after milking. The duct is lined with stratified squamous epithelium that produces a waxy layer of keratin capable of trapping bacteria, preventing their entry into the gland. This layer contains other components such as fatty acids, which prevent the growth of bacteria by binding electrostatically to their cell wall making them more susceptible to lysis by changes in osmotic pressure (Lacy-Hulbert & Hillerton, 1995). The proximal end of the teat canal contains a structure of 6-10 connective tissue folds covered with two layers of columnar epithelium, known as the Furstenberg's rosette. Structures resembling lymphoid follicles have been observed surrounding the Furstenberg's rosette in addition to lymphocyte infiltration. This suggests that the

Furstenberg's rosette not only functions as a physical barrier, but also plays an immunological role in the defence against invading bacteria (Aşti *et al*., 2011; Akers, 2016). If the bacteria are able to evade these properties, they enter the mammary gland cistern where they begin to colonise and proliferate in milk.

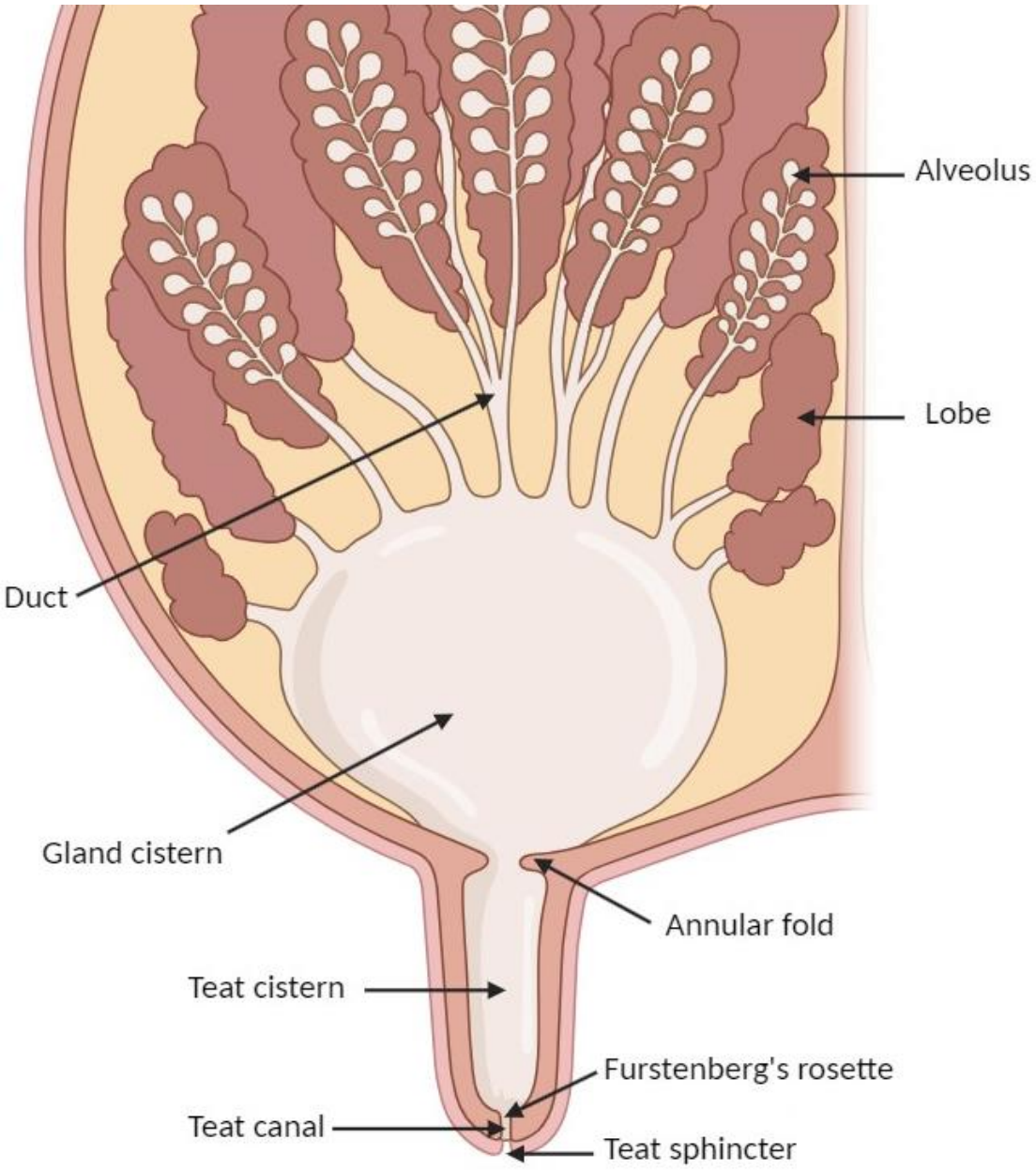


Fig 1.1. Anatomy of the bovine mammary gland. The teat sphincter functions as a physical barrier for the teat canal. The Furstenberg's rosette is situated at the proximal end of the teat canal and consists of 6-10 connective tissue folds covered with two layers of epithelium. The annular fold separates the teat and gland cistern. Lobes contain alveoli where milk is produced and enters the gland cistern via the ducts. Figure created using BioRender.com.

Unlike other environmental niches, bacteria in the mammary gland do not necessarily need to possess virulence factors that are considered aggressive. Instead, to colonise the mammary gland, bacteria may lose these aggressive attributes (as suggested in the case of *S. aureus*) and instead require passive fitness qualities such as iron acquisition systems and enzymes to degrade casein and lactose in the nutrient-rich milk (Herron-Olson *et al*., 2007; Richardson *et al*., 2018; Marbach *et al*., 2019).

Following entry of bacteria into the lactating bovine mammary gland, colonisation is dependent on replication in the extracellular environment (Smith *et al*., 2003). Growth of *E. coli* is evident as early as 4h, with peak bacterial counts of $1x10^7$ CFU (colony forming units)/mL detected at 16-24h post challenge. Increased *E. coli* growth corresponds with decreased milk lactose and glucose-6-phosphate at 12-16h post challenge (Blum *et al*., 2017 & 2020). Virulent *S. uberis* strains *in vivo* can reach in excess of 10^6-10^7 CFU/mL in milk. Less virulent, or attenuated strains, colonise less well (~10^3-10^4 fold) (Leigh *et al*., 2010; Egan *et al*., 2012; Tassi *et al*., 2013; Hossain *et al*., 2015). Establishment of a bacterial population within the mammary gland prior to host immune detection is required to prevent eradication. Once bacteria are detected, the host innate immune response is initiated.

1.2.2. Bovine mammary gland innate immune response

The innate immune response is the hosts' first line of defence against invading pathogens following penetration of the teat canal physical barrier. This non-specific response occurs quickly and involves activation and recruitment of immune cells to the site of infection within the lumen of the mammary gland. During intramammary infection, bovine mammary epithelial cells (bMECs) typically initiate the innate immune response. Depending on the location within the mammary gland, the epithelium consists of one or two layers of epithelial cells situated on a basement membrane (Rainard *et al*., 2022b).

At various stages of activity, macrophages migrate from udder tissue and can reside either between epithelial cells, called ductal macrophages, or in the lumen within milk, known as bovine mammary macrophages (BMMOs) (Mielke & Koblenz, 1981). Ductal macrophages are able to sample the lumen for the presence of bacteria; forming a communication network between the luminal and basal layers of the epithelium (Dawson *et al*., 2020) (Fig 1.2). The sub-epithelial stroma is heavily vascularised, readily allowing for the recruitment of immune cells, such as neutrophils and lymphocytes, to the site of infection (Rainard *et al*., 2022c). It is important to understand how bMECS, BMMOs and neutrophils interact to collectively initiate the innate immune response to invading bacterial pathogens within the mammary gland.

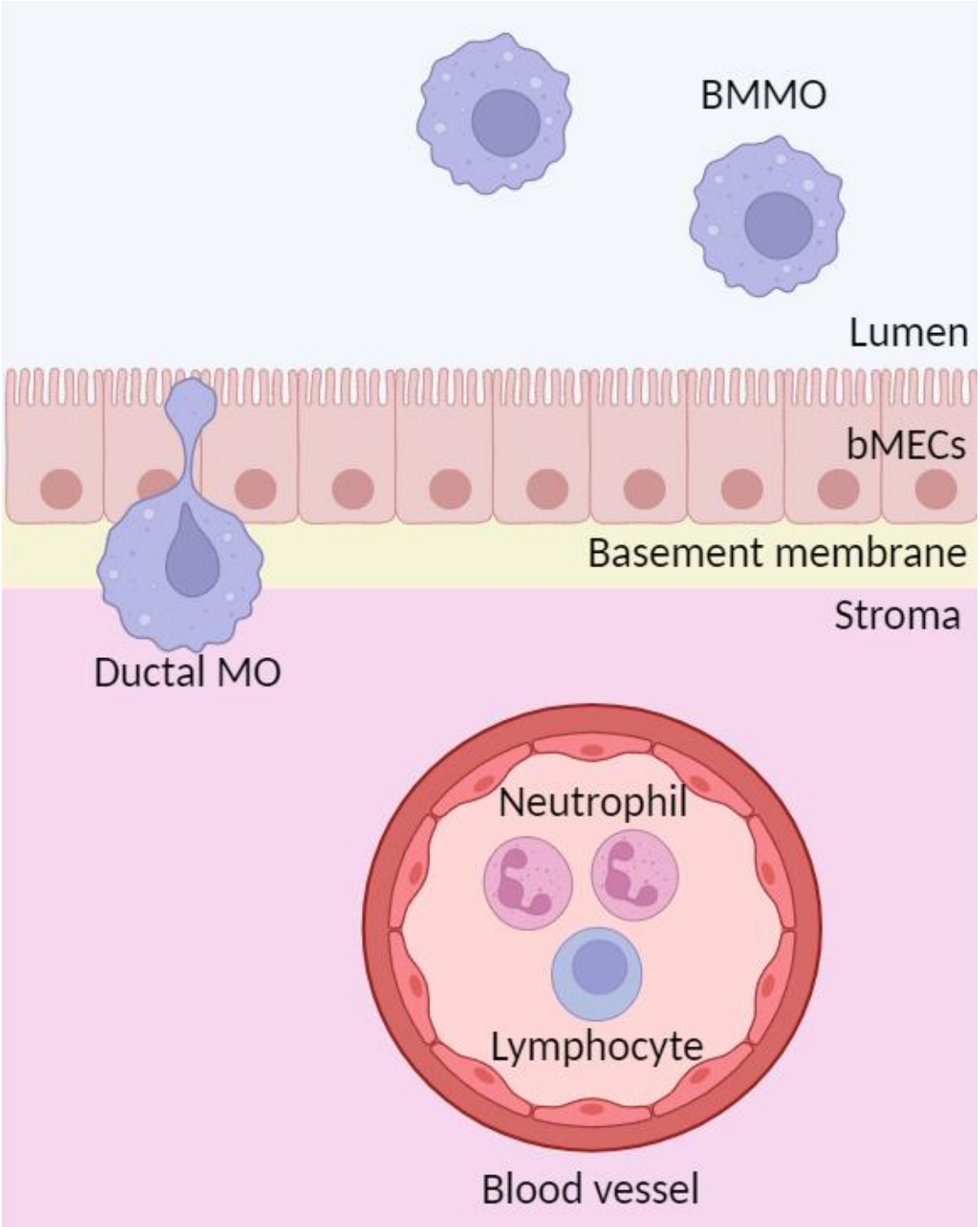


Fig 1.2. Bovine mammary gland epithelium. Mammary gland epithelium consists of a layer of bovine mammary epithelial cells (bMECs) and a basement membrane. Macrophages (MO) are situated between epithelial cells, known as ductal MO, or in the mammary gland lumen within milk, called bovine mammary macrophages (BMMO). Blood vessels are present in the stroma and contain immune cells such as neutrophils and lymphocytes. Figure created using BioRender.com.

1.2.2.1. Bovine mammary epithelial cells

Bovine MECs initiate the innate immune response through bacterial pathogen recognition. As bacteria are within the lumen of the mammary gland, bMECs express mucins, most notably MUC1, promoting adherence of *E. coli* and *S. aureus* to the epithelium to gain proximity for

recognition (Patton *et al*., 1995; Sando *et al*., 2009). MECs express pattern-recognition receptors (PRRs) which bind to pathogen associated molecular patterns (PAMPs) on bacteria (Rainard *et al*., 2022b). There are six classes of PRRs, of which, toll-like receptors (TLRs) sense bacteria at the extracellular surface and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) detect bacteria in the cytosol (Benko *et al*., 2008). An abundance of TLR2 and TLR4 mRNA was found in severely infected quarters compared to uninfected control quarters from the same cow (Goldammer *et al*., 2004). Bovine MECs recognise *E. coli* through TLR4 binding to lipopolysaccharides (LPS) and *S. aureus* through TLR2 to lipoteichoic acid (LTA) (Ibeagha-Awemu *et al.,* 2008; Ray *et al*., 2013). These interactions are enhanced through the expression of co-receptors. CD36 is a co-receptor that aids TLR4 recognition of *E. coli* (Cao *et al*, 2016). The accessory molecule CD14 (cluster of differentiation 14) facilitates recognition of both Gram-positive and Gram-negative bacteria and is either membrane bound or soluble. In the bovine mammary gland, there was no expression of CD14 on MECs, but soluble CD14 was detected at increased concentration during mammary gland inflammation (Lee *et al*., 2003). Recognition of bacteria by bMECs results in the production of inflammatory cytokines and chemokines. This response varies, depending on the bacteria interacting with the bMECs.

In response to *E. coli*, bMECs were found to activate the transcription factor NF-κB (nuclear factor kappa B) and produce the pro-inflammatory cytokines IL-1α (interleukin-1 alpha), TNF-α (tumour necrosis factor alpha) and the chemokine IL-8 (Boudjellab *et* al., 1998; McClenahan *et al*., 2005 & 2006; Yang *et al*., 2008). MECs were also found to produce TNF-α and IL-8 following stimulation with *S. aureus*. However, these inflammatory mediators were produced to a lesser extent and lacked NF-κB activation when compared to stimulation with *E. coli* (Yang *et al*, 2008; Xu *et al*., 2019). The bMEC response to *S. aureus* additionally resulted in the production of IL-6 and IFN-β (interferon beta) (Günther *et al*., 2011). Secreted cytokines interact with and activate neighbouring cells, enhancing the inflammatory response.

Contrary to most *E. coli* strains, *S. aureus* can adhere and internalise into bMECs to evade the bovine immune response (Almeida *et al*., 1996a). However, invasion of the bovine mammary epithelial cell line (MAC-T cells) by *S. aureus* activates the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome resulting in pyroptosis and the release of the pro-inflammatory cytokines IL-1β and IL-18 (Almeida *et al*., 1996a; Wang *et al*., 2022). In addition to IL-8, stimulated bMECs also secrete the chemokine CCL20 (C-C motif chemokine ligand 20). Together, IL-8 and CCL20 bind to receptors on neutrophils and lymphocytes to recruit these immune cells to the infected mammary gland

(Petzl *et al*., 2016). Recognition of bacterial pathogens and recruitment of immune cells is also facilitated by macrophages.

1.2.2.2. Macrophages in the mammary gland

Macrophages are the predominant leukocytes found in a healthy mammary gland, constituting 10-80% of the total leukocyte population (Ostensson *et al*., 1988; Paape *et al*., 2002; Schwarz *et al*., 2011). BMMOs have three main functions in response to bacterial intramammary infection (Fig 1.3). Similar to bMECs, BMMOs express PRRs (including TLR2 and TLR4) which recognise and bind to bacterial PAMPs, triggering a signalling cascade that activates NF-κB (Gunther *et al.,* 2016a). Alternatively, macrophages sample the extracellular environment as a method for immune surveillance (Paape *et al*., 2000). Activated BMMOs phagocytose and digest bacterial cells via proteases and reactive oxygen species (ROS) (Mullen *et al*., 1985; Gong *et al*., 2018). BMMOs from lactating cows were found to be able to kill a significant proportion of *S. aureus*. However, mammary macrophages failed to efficiently kill *S. uberis* (Denis *et al*., 2006; Sacco *et al*., 2020).

Ultimately, BMMOs secrete pro-inflammatory mediators including TNF-α, IL-1β and IL-8 to activate neighbouring immune and epithelial cells and recruit neutrophils and lymphocytes to the site of intramammary infection (Denis *et al*., 2006). Production of cytokines, such as IL-1β, is facilitated in macrophages through the priming and activation of the NLRP3 inflammasomal pathway (Quinton *et al*., 2007). BMMOs also branch the innate and adaptive immune systems through antigen presentation to lymphocytes, such as T-cells, via MHCII (major histocompatibility complex class II) (Denis *et al*., 2006).

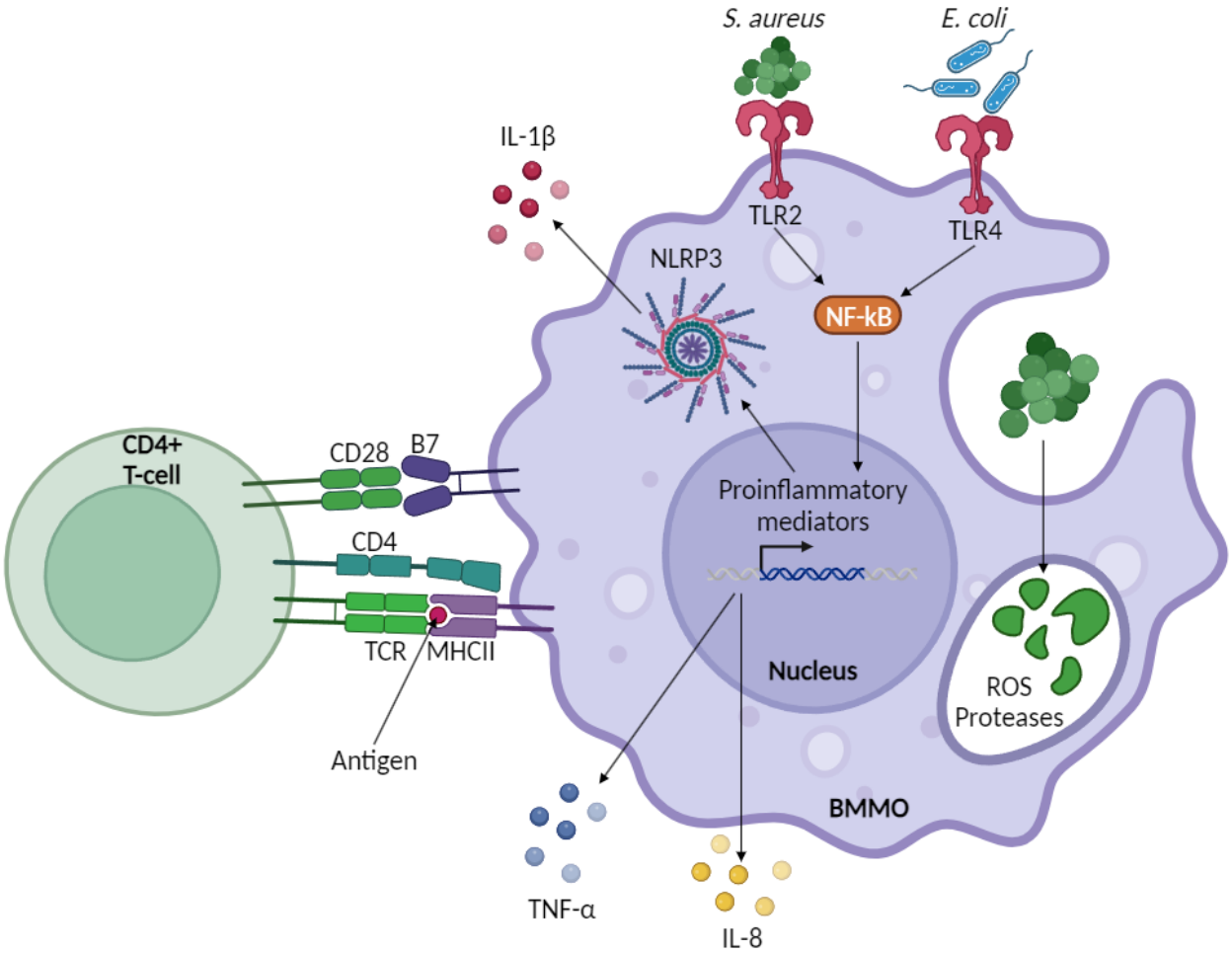


Fig 1.3. Immune functions of bovine mammary macrophages. Bovine mammary macrophages (BMMOs) recognise S. aureus and E. coli pathogen associated molecular patterns via toll-like receptor (TLR) 2 and 4 respectively. This leads to a signalling cascade that activates the transcription factor NF-κB, which translocates into the nucleus and upregulates expression of pro-inflammatory mediators including TNF-α, IL-8 and IL-1β. These mediators are secreted out of the BMMO to stimulate an immune response through the activation of neighbouring epithelial and immune cells. Prior to secretion, IL-1β is processed by the NLRP3 inflammasome. BMMOs also phagocytose bacteria either through recognition or via immune surveillance. Bacteria are digested with the phagolysosome by reactive oxygen species (ROS) and proteases. Bacterial antigens are presented to CD4+ T-helper cells via MHCII and T-cell receptor (TCR) interactions. This is facilitated by the co-stimulatory molecule CD4 and the interaction between CD28 and B7. Figure created using BioRender.com

1.2.2.3. Neutrophils

Neutrophils constitute 3-26% of the total leukocytes in healthy bovine mammary gland milk, but during infection neutrophils become the predominant cell type at ~90% (Harmon, 1994; Kehrli & Shuster, 1994; Alnakip *et al*., 2014). This significant increase occurs because following pathogen recognition, neutrophils are recruited to the site of infection within the mammary gland to complete their bactericidal activity (Fig 1.4).

Stimulated bMECs and macrophages secrete chemokines, such as IL-8, basolaterally, creating a concentration gradient between the epithelium and blood vessels. TNF-α causes vasodilation of the blood vessels surrounding the infected mammary gland and upregulation of adhesion molecules expressed on endothelial cells. Increased blood flow provides the opportunity for a greater number of neutrophils to bind selectins and integrins and traverse the endothelium in a process known as diapedesis (Paape *et al*., 2000; Rainard & Riollet, 2003). In the bovine mammary gland, diapedesis was found to be dependent on β_2-integrin function (Smits *et al*, 2000). When neutrophils enter the subepithelial space, they follow the chemokine gradient to the epithelium and cross the tight junction to enter the mammary lumen (Lee & Zhao, 2000).

In bovine mammary glands it was found that there was an increase in the percentage of neutrophils after challenge with *E. coli* (Hill, 1981). Only 0.2 µg of LPS was needed to trigger the influx of neutrophils into the lactating mammary gland, despite the dilution in 40 mL residual milk (Rainard *et al*., 2016). Although LTA also triggered neutrophil influx, much higher concentrations were required compared to LPS (Rainard *et al*., 2008).

Once neutrophils are recruited to the mammary gland, they are able to phagocytose bacteria. Phagocytosis has been reported as relatively efficient as most strains of *E. coli* are not resistance to neutrophil killing. This was shown as bacterial numbers reduced to approximately 20% within 1h of incubation with bovine neutrophils (Roussel *et al*., 2017). However, the high content of fat globules and casein micelles within milk interfere with neutrophils ability to phagocytose and kill (Paape & Guidry, 1977). Neutrophil bactericidal activity has also been shown to be impaired due to the low oxygen tension in milk (Goldberg *et al*., 1995).

Phagocytosed bacteria are killed by proteolytic enzymes, antimicrobial proteins and ROS within neutrophils (Brinkmann *et al*., 2004). Additionally, neutrophils degranulate, releasing antimicrobial factors into the surrounding extracellular environment to aid bactericidal activity. Neutrophils have also been shown to produce extracellular traps (NETs) in response to both Gram-positive and Gram-negative bacteria (Brinkmann *et al*., 2004). NETs comprise of a DNA framework with proteins including histones, elastase and myeloperoxidase attached. These

structures can act as a physical barrier to prevent the spread of bacteria but also prevent the spread of granular contents while still effectively killing pathogens (Papayannopoulos & Zychlinsky, 2009). Formation of NETs were found in milk neutrophils during clinical mastitis *S. aureus* infection, whereas NET formation was not evident during subclinical mastitis. NET formation was found to correlate in clinical mastitis with an increased expression of TLR2 and TLR4 on BMMOs (Swanson *et al*., 2014).

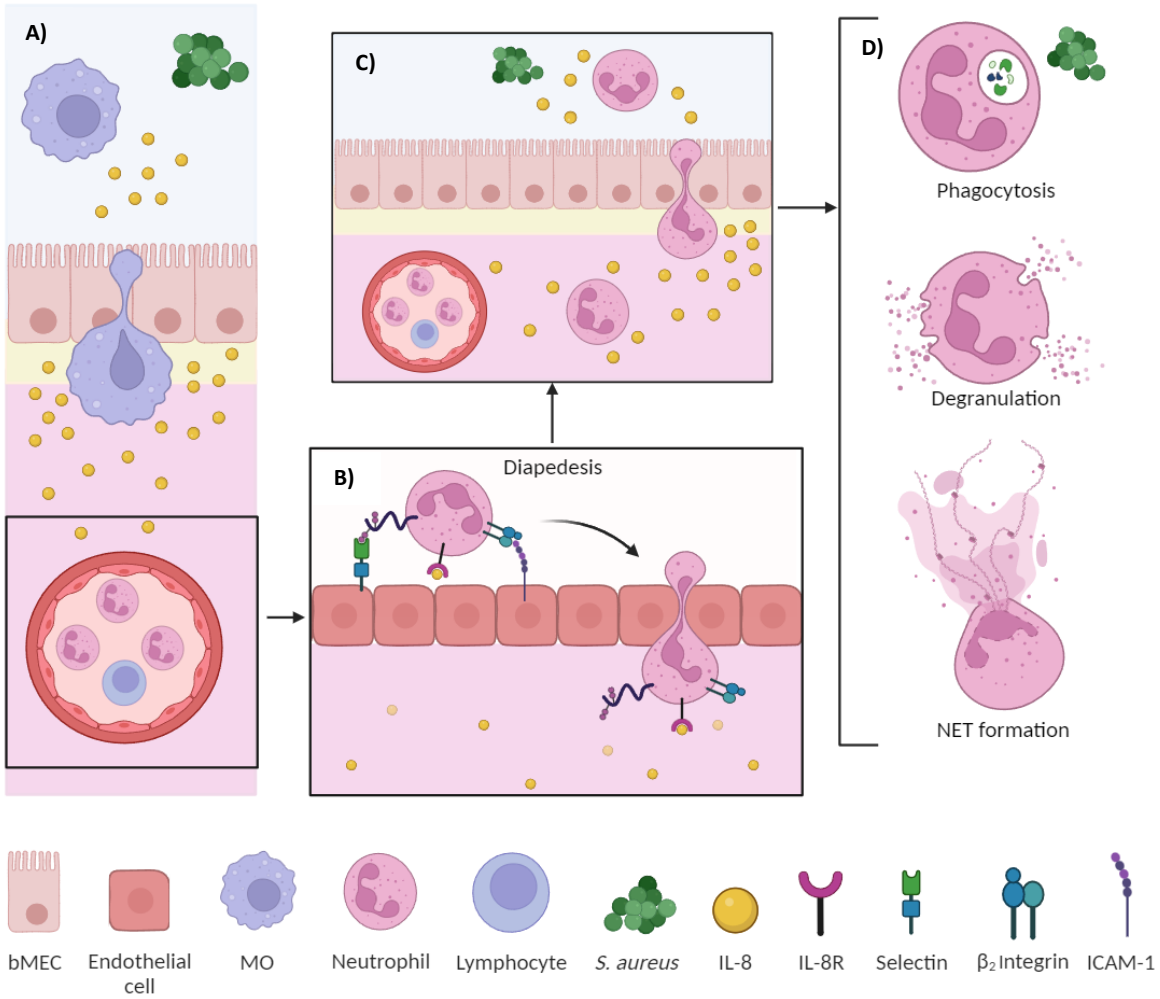


Fig 1.4. Neutrophil recruitment and function in the bovine mammary gland. A) Activated bovine mammary epithelial cells (bMECs) and macrophages secrete the chemokine IL-8, which stimulates blood vessels to increase expression of adhesion markers (selectins, β2 integrins and ICAM-1). **B**) Neutrophils within the blood vessels bind to adhesion markers and traverse the endothelium into the subepithelial space in a process called diapedesis. **C**) Neutrophils follow the IL-8 concentration gradient and cross the tight junctions between epithelial cells, arriving at the site of infection within the mammary gland. **D**) Bacteria are either phagocytosed and killed, neutrophils degranulate or form neutrophil extracellular traps (NETs) releasing bactericidal proteins. Figure created using BioRender.com.

1.2.2.4. Other components

In addition to immune cells, other components within bovine milk help the antimicrobial response to invading pathogens. An example of this is lactoferrin (LF), an iron-binding glycoprotein produced by secretory epithelial cells in the mammary gland (Molenaar *et al*., 1996). Transferrin (TF) is another iron-binding protein. However, unlike LF, TF is not synthesised in milk. Instead, it is produced in the blood and enters the mammary gland during plasma exudation as a consequence of mastitis (Ollivier-Bousquet, 1998). Both LF and TF compete with bacteria for available iron and can bind to LPS on *E. coli*, damaging the outer membrane resulting in altered cell integrity and cell wall permeability (Erdei *et al*., 1994). LF can also bind to *S aureus*, *S. agalactiae* and *S. uberis* preventing adherence and invasion into bMECs (Naidu *et al*., 1991; Rainard, 1992; Moshynskyy *et al*., 2003). Effects of LF are dependent on the iron requirements of the bacteria, i.e., *E. coli* are most susceptible and streptococci are most resistant (Rainard, 1987).

Three important antimicrobial enzymes present in bovine milk are lysozyme, lactoperoxidase and myeloperoxidase. Lysozyme cleaves the β 1,4-glycosidic bond between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan in Gram-positive bacteria, disrupting the cell wall (Priyadarshini & Kansal, 2002). Alone, lysozyme bactericidal activity is insufficient at clearing bacterial infections; its actions synergise with antibodies, complement and LF (Sordillo LM & Streicher, 2002; Rainard & Riollet, 2006). The antimicrobial peptide β-defensins 1, 4 and 5 are expressed in mammary gland tissue and also interact with bacterial membranes causing their destabilisation (Selsted *et al*., 1993; Swanson *et al*., 2004). Lactoperoxidase levels start to increase 12h post *E. coli* challenge and exert bactericidal activity via the generation of activated oxygen products, such as hypothiocyanite, which promotes neutrophil activity (Blum *et al*., 2020). Myeloperoxidase is located in primary granules of neutrophils and catalyses the same reaction as lactoperoxidase but also catalyses the oxidation of chloride. Unlike lactoperoxidase, the antimicrobial properties of myeloperoxidase are inhibited by milk proteins during the lactation period and so only play a role in the immune response in the dry period (Cooray & Björck, 1995; Sordillo LM & Streicher, 2002).

The alternative pathway of the complement system has been detected in the mammary gland due to increased C3 mRNA expression in bMECs during *E. coli* and *S. aureus* intramammary infections (Griesbeck-Zilch *et al*., 2008). This pathway utilises C3b opsonisation of bacteria (increasing recognition and phagocytosis by neutrophils) and the pro-inflammatory mediator C5a (Rainard & Poutrel, 1995; Rainard *et al*., 1998). *S. aureus* produces extracellular fibrinogen

binding protein during intramammary infection which inhibits the complement system by preventing C3 deposition on the bacterial surface (Lee *et al*., 2004).

Despite the antibacterial effectiveness of these components, they are insufficient at mounting an immune response alone. This may be due to their insufficient concentrations following daily milking and draining of the mammary gland. Together, bMECs, macrophages, neutrophils and accessory components act quickly and non-specifically to initiate the immune response. In order to fully clear intramammary infections, macrophages communicate with and stimulate a specific adaptive immune response.

1.2.3. Bovine mammary adaptive immune response

The adaptive immune response is comprised of two main cell types, T-cells and B-cells. Together, in conjunction with interactions with cells of the innate immune response, pathogens are cleared from the site of infection in a specific manner. The adaptive immune response can result in immunological memory of the invading pathogen. Therefore, subsequent reinfections are recognised as soon as the pathogen invades, resulting in faster bacterial clearance, limiting host damage.

1.2.3.1. T-cells

Following T-cell production and development in the thymus, naïve T-cells are situated locally to the mammary gland and circulate the secondary lymphoid tissue (e.g., lymph nodes and surrounding vasculature). Upon initiation of the innate immune response, naïve T-cells migrate into the subepithelial space surrounding the site of infection via the same diapedesis mechanism as neutrophils. Here, T-cells become effector cells where they are no longer confined to the lymphatic system and can migrate throughout the mammary gland (Butcher & Picker 1996). Studies have demonstrated that the migratory properties of bovine mammary T-cells are different from non-ruminant species. Radiolabelled mammary lymph node T-cells are localised to mammary and peripheral lymph nodes but not to intestinal mesenteric lymph nodes and *vice versa* (Harp *et al*., 1988). This was found to be due to the high expression of the peripheral node homing receptor CD62L on bovine mammary T-cells (Bosworth *et al*., 1993). These results suggest that the mammary immune system is not linked to the intestinal immune system and mammary T-cells originate from peripheral rather than mucosal sites. This could explain why bacteria act commensally in the gut but become opportunistic pathogens in the mammary gland as these immune systems are not in communication with each other.

Following migration of naïve T-cells into the mammary gland tissue, activated macrophages process and present bacterial antigens to CD4+ T helper (Th) cells via MHCII (Fig 1.3) (Denis *et al*., 2006). Th cells express T-cell receptors (TCR) and CD4 co-receptors which bind to different sections of the MHCII molecule, resulting in a signalling cascade that provides the T-cell activation signal (Fig 1.3). This is followed by a second signal involving the interaction between co-stimulatory molecules CD28 on CD4+ T-cells and B7 (CD80/86) on macrophages, verifying T-cells are responding to foreign antigens. These two signals promote secretion of IL-2 from T-cells which act upon themselves, promoting proliferation. Finally, depending on the cytokines present in the environment, CD4+ T-cells differentiate into one of the five major Th subsets (Th1, Th2, Th17, Treg or Tfh). Each Th subset dictates a specific type of adaptive immunity which is dependent on the cause of the immune response (Murphy & Weaver, 2016; Jeffery, 2023).

Recent evidence has outlined the importance of Th17 cells during intramammary infections following the isolation and expanded culture of bovine Th17 cells (Cunha *et al*., 2019). Th17 cells are characterised by their secretion of the pro-inflammatory cytokines IL-17A, IL-17F and IL-22. These cytokines mediate protective immunity against fungi and bacteria by promoting neutrophil recruitment, antimicrobial peptide production and enhance barrier function (Mills, 2023). Bovine mammary gland milk leukocytes were found to have increased expression of IL-17A, IL-17F and IL-22 following *E. coli*, S. *aureus* and *S. uberis* infection as soon as 8h post-challenge (Riollet *et al*., 2006; Tao & Mallard, 2007; Bruno *et al*., 2010; Rainard *et al*., 2013; Tassi *et al*., 2013; Roussel *et al*., 2015). IL-17 receptors, IL-17RA and IL-17RC, are expressed on bMECs. Corresponding cytokine binding to IL-17Rs amplifies the immune response when simultaneously stimulated with staphylococcal PAMPs by enhancing secretion of IL-18 and TNF-α from bMECs (Bougarn *et al*., 2011). These data suggest that intramammary infections promote the differentiation of Th17 cells which produce IL-17, IL-17F and IL-22 cytokines that recruit neutrophils and stimulate bMECs antibacterial defences.

Cytotoxic CD8+ T-cells become activated following interactions with antigens presented on MHCI and function by killing cells that are infected with intracellular bacteria. Involvement of CD8+ T-cells during intramammary infections has not been as thoroughly investigated as the Th cell response. Previous studies found that increasing the dose of *E. coli* accelerated trafficking of CD8+ T-cells to the bovine mammary gland, suggesting their involvement in the immune response (Mehrzad *et al*., 2008). It has been previously demonstrated that *S. aureus* infects bMECs, however, there is no evidence that indicates CD8+ T-cells interact with MHCI expressed on bMECs despite immunohistochemistry analysis concluding CD8+ T-cells are

situated in close proximity to bMECs during intramammary infections (Yamaguchi *et al*., 1999; Halle *et al*., 2017). Further investigation is required to determine the role of CD8+ T-cells in bovine mastitis.

1.2.3.2. B-cells

CD4+ T-cells interact and activate B-cells which function as antigen presenting cells and differentiate into either plasma cells that produce antibodies or memory cells (Murphy & Weaver, 2016). IgG is regarded as the staple antibody against bovine bacterial intramammary infections. IgG functions in the immune response by opsonising bacteria to aid in their recognition and ingestion by immune cells (Burton & Erskine, 2003; Zhang *et al*., 2023).

Mastitis has been reported to not induce protection through immune memory. For example, an udder quarter infected with *S. aureus* that was cleared following treatment and displayed an increase in specific antibodies could still be reinfected with the same strain (Rainard *et al*., 2022a). The same was found following mammary gland infection with *E. coli*. Initial infection induced opsonic antibodies in milk, however, there was no evidence of protection as the extent of disease remained the same in recurrent infections (Hill *et al*., 1983). However, some studies have shown evidence for immune memory. In one study, *E. coli* intramammary infection was cleared rapidly when cows were challenged with the same strain 150 days later (Hill, 1981). Therefore, the effectiveness of bovine B-cells and antibodies in immune memory to intramammary infections remains unclear.

1.3. Intramammary infection with *S. uberis*

S. uberis is a Gram-positive, catalase-negative bacterium and a member of the *Streptococcaceae* family. It is the most prevalent intramammary pathogen in the UK and is becoming increasingly important globally (Bradley *et al*., 2007; UK-VARSS, 2019). *S. uberis* can be considered a commensal within cattle and is commonly found in the gastrointestinal tract, but has also been isolated from the skin, tonsils and genital tract (Bradley *et al*., 2007; Klaas & Zadoks, 2018; Keane, 2019). However, when *S. uberis* gains access to the mammary gland it functions as an opportunistic pathogen. It is most likely that *S. uberis* infects the mammary gland from environmental sources. Absence of cattle on pasture for approximately 3 weeks decreased *S. uberis* to almost undetectable levels indicating that the likelihood that reseeding of the environment is required; the most likely source of which being cattle faeces (Zadoks *et al*., 2005; Lopez-Benavides *et al*., 2007). Previous studies have outlined how *S. uberis*

pathogenesis differs compared to other intramammary pathogens. These differences have been investigated through the exploitation of mutants.

1.3.1. S. uberis virulence determinants and their impact on the immune response

The genome of *S. uberis* is the smallest across the *Streptococcus* family with a single circular chromosome that is between 1.8-2.3 Mb (Ward *et al*., 2009; Davies *et al*., 2016). Multi locus sequence typing has identified over 900 sequence types of *S. uberis* with different virulence exhibited by differing strains. The 0140J strain is the most studied and has been used in many experiments to investigate pathogenesis as it is regarded as virulent. In contrast, the strain EF20 has been described as avirulent (Hill, 1988; Hossain *et al*., 2015). Studies have compared the virulence phenotype between these two strains. Virulence genes have been proposed by sequencing of the 0140J strain, however, whole genome analysis found many of these were also found in the EF20 strain. Therefore, the presence/absence of known virulence genes is insufficient to determine the outcome of infection. Analysis of thirteen *S. uberis* strains (including 0140J and EF20) identified a core genome of 1,550 shared genes (Hossain *et al*., 2015). Further investigations discovered virulence determinants of interest that are associated with the bacterial cell surface. These determinants are involved in adherence, colonisation and evasion of the host immune response during *S. uberis* intramammary infection (summarised in Table 1.1) (Egan *et al*., 2010; Leigh *et al*., 2010).

1.3.1.1. S. uberis sortase A (SUB0881)

In Gram-positive bacteria, many surface proteins have been reported to be covalently anchored to the cell wall via the transamidase, sortase (Schneewind *et al*., 1992). Substrate proteins contain a hydrophobic region and a short tail of charged amino acids at the C-terminus which retain proteins at the bacterial cell surface. Anchoring of proteins involves the cell wall sorting motif LPXTG. Sortase cleaves this motif resulting in the formation of cross-bridges within the peptidoglycan to stabilise the proteins on the cell surface (Perry *et al*., 2002).

In *S. uberis* strain 0140J, the transpeptidase sortase A (SrtA; SUB0881) anchors a specific and discrete subset of proteins to the cell surface. It is hypothesised that SUB0881 mutants would fail to anchor the substrate proteins to the cell surface. Egan *et al*., 2010, exploited this to identify nine coding sequences (CDSs) displaying characteristics of SrtA anchored proteins by comparing the proteome of cell walls purified from *S. uberis* strain 0140J and an isogenic mutant lacking SUB0881. Five of these substrates were found to contain typical (LPXTG) anchor motifs: SUB0145 (lactoferrin binding protein; Lbp), SUB0207, SUB0888, SUB1095 (collagen like

surface anchored protein; SclB) and SUB1730. The other four (SUB0135, SUB0826, SUB1154 (serine protease; ScpA) and SUB1370) contained an atypical (LPXXXD) sorting motif.

Leigh *et al*., 2010, investigated the role of sortase and the other sortase substrate proteins by comparison of the virulence of mutants lacking SrtA and the individual SrtA substrate proteins. This study found that although the mutant SUB0881 colonised at a rate similar to the parental strain, 0140J, for the first 24h post challenge, the mutant was unable to colonise to high levels and failed to induce signs of disease despite there also being a similar speed and magnitude of cellular infiltration in response to infection. These data indicated that the anchoring of proteins by SUB0881 was required for high level colonisation and the induction of clinical signs of disease. Further analysis of the nine SrtA anchored proteins found that mutants lacking SUB0145, SUB1095 and SUB1154 were attenuated as they failed to induce clinical mastitis. Therefore, these three proteins were suggested to play an important, independent role in colonisation and the pathogenesis of mastitis as their expression was required for full virulence. These data concluded that SrtA (SUB0881) was important for virulence as it enabled correct localisation of at least three virulence determinants.

1.3.1.2. S. uberis lactoferrin binding protein (SUB0145)

The gene product from *sub0145* has been reported to be a protein capable of binding LF (Moshynskyy *et al*., 2003). During intramammary infection there is an increase in bovine milk of the iron-binding glycoprotein, LF, as part of the host immune response. Iron chelation is a known antimicrobial mechanism by limiting free iron availability to invading pathogens (Harmon *et* al., 1976; Hagiwara *et al*., 2003). LF has been shown to inhibit growth of *S. aureus* and *E. coli* causing mastitis pathogens. However, LF had no effect on *S. uberis* cell growth even in iron-depleted growth media, indicating that restricting iron acquisition is not inhibitory for growth of *S. uberis* (Moshynskyy *et al*., 2003; Chaneton *et al*., 2008). Some studies have shown that in fact, LF enhances adhesion of *S. uberis* to host cells, increasing invasiveness (Fang *et al*., 2000). Alternatively, the acquisition of manganese has been shown to be critical for *S. uberis* growth in milk, through analysis of mutation in the *mtuA* gene (metal transporter uberis A) and mastitis in dairy cattle. The MtuA mutant, AJS001, was unable to grow in milk without the addition of exogenous manganese or infect bovine mammary glands as there was no evidence of an inflammatory response or signs of mastitis (Smith *et al*., 2003). This indicates that in the absence of MtuA, *S. uberis* is totally avirulent, suggesting the importance of manganese for bacterial growth.

Despite SUB0145 not being required for growth in iron-limiting environments, mutations in SUB0145 have been shown to impair virulence, suggesting SUB0145 possesses an alternative function. Fenske *et al*., 2022, found that SUB0145 was present in all 24 *S. uberis* isolates examined, further suggesting the importance of SUB0145. Crowley *et al*., 2011, investigated the differential protein expression in *S. uberis* under planktonic and biofilm growth conditions and found that the transition from planktonic to biofilm growth coincided with increased expression of SUB0145. However, there was decreased expression of SUB0145 in 36h biofilms compared to 8h biofilms, indicating a possible role for SUB0145 in the formation, but not the maintenance of biofilms.

Additionally, the ability for bacterial pathogens to adhere and become internalised into the host cell is often important for colonisation and persistence of infection through evasion of the host immune response. LF has been suggested to act as a bridging molecule that facilitates close proximity of *S. uberis* to bMECs through LF binding receptors expressed on bMECs (Patel *et al*., 2009). *S. uberis* is also capable of transcytosing bMECs and persisting in the cytoplasm for up to 120h, evading pathogen recognition and host defences (Almeida & Oliver, 2006). Adherence experiments utilising SUB0145 mutants however suggest that SUB0145 is not required for *S. uberis* attachment to bMECs. The predicted protein sequence of SUB0145 has a 28% homology to streptococcal M proteins, which prevent phagocytosis by inhibiting bacterial opsonisation. Therefore, SUB0145 may facilitate internalisation into bMECs rather than attachment (Ward *et al*., 2009).

1.3.1.3. S. uberis adhesion molecule (SUB1635)

Alternatively, *S. uberis* has been found to express the *S. uberis* adhesion molecule (SUAM; SUB1635), which possesses affinity for bovine milk LF (Almeida *et al*., 2006). Antibody studies have found that antibodies against SUB1635 and LF inhibit adherence and internalisation of *S. uberis* into bMECs (Almeida *et al*., 2006 and Patel *et al*., 2009). The *Sua* gene mutant in *S. uberis* UT888 was found to be unable to express SUB1635. *In vitro* analysis showed that mutant SUB1635 had reduced adherence and internalisation into bMECs. The effect of SUB1635 was further analysed *in vivo*. It was found that fewer mammary glands became infected and there was reduced clinical signs of mastitis in the mutant compared to the wild type (Almeida *et al*., 2015). These data suggest that SUB1635 is involved in the colonisation of *S. uberis* in the mammary gland through adherence to bMECs via LF and in persistence of infection as *S. uberis* is internalised into bMECs evading host defences. Recombinant SUB1635 was tested as a potential *S. uberis* vaccine. Immunised cows produced antibodies that reduced adherence and

internalisation of *S. uberis* into MAC-T cells (Prado *et al*., 2011). However, when tested *in vivo*, vaccination with SUAM was not protective as all cows developed mastitis (Siebert *et al*., 2017).

1.3.1.4. S. uberis collagen-like surface protein (SUB1095)

S. pyogenes was found to express the collagen-like surface protein, SclB, which was later found to share a 46% homology with *S. uberis* SUB1095 (Whatmore, 2001; Leigh *et al*., 2010). In *S. pyogenes*, SclB functions by adhering to fibroblasts and binding to host thrombin-activatable fibrinolysis inhibitor to modulate the host response to bacterial infection (Pahlman *et al*., 2007). Additionally, in *S. pyogenes*, SclB binds to $\alpha_2\beta_1$ integrins to facilitate adherence and internalisation to evade the immune response (Caswell *et al*., 2007). $\alpha_2\beta_1$ integrins are expressed on fibroblasts, NK cells and platelets and have more recently been found on Th1 and Th17 cells (Abderrazak *et al*., 2018). Although the function of SUB1095 in *S. uberis* remains unknown, mutants lacking SUB1095 resulted in reduced colonisation after 24h compared to the wild type (Leigh *et al*., 2010).

1.3.1.5. S. uberis positive transcriptional regulator Vru (SUB0144)

Egan *et al*., 2012, investigated the role of SUB0144 (Vru) in bovine challenge studies and the effect of its absence on transcriptional control of gene expression by microarray analysis. In contrast to the wild-type strain, the SUB0144 mutant showed poor colonisation of the mammary gland and failed to induce signs of mastitis. It was found that compared to the wild type, there was reduced expression of SUB0145, SUB1095, SUB1697 (HasA; capsule biosynthesis protein) and SUB1785 (PauA; plasminogen activator) in the absence of SUB0144. This confirms that SUB0145 and SUB1095 are among the most important proteins involved in virulence. Despite Mga controlling expression of ScpA in *S. pyogenes*, it was shown that SUB0144 did not regulate the expression of SUB1154 in *S. uberis*. Similarly to SUB0881, a single mutation or ablated expression of SUB0144 resulted in the downregulation of several genes resulting in reduced intramammary colonisation and pathogenesis.

1.3.1.6. S. uberis hyaluronic capsule and biofilm formation

The hyaluronic acid capsule of a number of streptococcal species has been implicated in disease pathogenesis, linked to invasion, adherence and evasion of phagocytic host defences. SUB1697 (HasA) is a hyaluronan synthase involved in the production of the capsule and is regulated by SUB0144 (Egan *et al*., 2012). Studies have investigated the *S. uberis* strain 0140J SUB1697 mutant, which was unable to produce a capsule, to determine the effect of the capsule on virulence.

Both encapsulated and non-encapsulated *S. uberis* strains were found to adhere to the bMEC line (MAC-T) and to extracellular matrix proteins (Almeida *et al*., 1996b). Other *S. uberis* strains have also been shown to adhere and become internalised into MAC-T cells, where they persist for up to 120h without losing viability. These strains were also reported to form biofilms (Tamilselvam *et al*., 2006; Fessia *et al*., 2020). Biofilm formation and internalisation into host cells allows bacteria to persist under environmental stress within the bovine mammary gland and avoid host immune recognition. Specifically, the α- and β-casein components in milk were reported to induce *S. uberis* biofilm formation (Varhimo *et al*., 2011). The *S. uberis* gene with homology to the oligopeptide permease gene of *S. pyogenes*, oppF, was found to be essential for the acquisition of the amino acid methionine through the utilisation of β-casein for growth in milk (Smith *et al*., 2002). In *vitro* studies also demonstrated that acapsular *S. uberis* were more susceptible to phagocytosis by bovine neutrophils as they had lost resistance due to the absence of the capsule (Ward *et al*., 2001). However, *in vivo* studies found that the wild type and SUB1697 mutant both resulted in clinical signs of disease with similar bacterial and SCC numbers observed in milk. These data indicate that the capsule does not play a role in resistance to the bactericidal effects of neutrophils *in vivo*. Although the *hasAB1* gene cluster is not present universally in *S. uberis*, it has been reported to be over-represented in isolates from clinical intramammary infections. This suggests the capsule could be important in another mechanism of pathogenesis and it has been hypothesised that the capsule may be involved in environmental survival and teat end colonisation and/or penetration (Field *et al*., 2003).

UBAC is the only commercially available vaccine targeting *S. uberis* mastitis. This vaccine utilises the *S. uberis* biofilm adhesion component (BAC) as an antigen to provoke an immune response. UBAC vaccination was found to significantly reduce milk yield losses and clinical signs of mastitis (Collado *et al*., 2018).

1.3.1.7. S. uberis plasminogen activator PauA (SUB1785)

The serine protease SUB1785 (PauA) can activate bovine plasminogen to plasmin, which then becomes associated with the bacterial surface (Leigh & Lincoln, 1997). It was hypothesised that bound plasmin, which was more resistant to physiological protease inhibitors (Lincoln & Leigh, 1998), was able to degrade host proteins into small peptides and free amino acids that can be used by *S. uberis* as a source of amino acids for this auxotrophic species (Kitt & Leigh, 1997; Smith *et al*., 2002). It was further hypothesised that SUB1785 may be involved in colonisation of the bovine mammary gland by facilitating bacterial growth *in vivo* (Leigh, 1993). This was supported by data using SUB1785 as a protective vaccine antigen. It was found that

dairy cows vaccinated with this protein produced an inhibitory antibody response and following challenge with *S. uberis* had lower levels of intramammary bacterial colonisation and exhibited reduced signs of disease (Leigh *et al*., 1999). However, studies using isogenic mutants demonstrated that SUB1785 was not required during colonisation or virulence of *S. uberis*, as a mutant lacking SUB1785 (unable to activate bovine plasminogen) was equally able to colonise and induce bovine mastitis as the parental, genetically intact strain (Ward *et al*., 2003). It remains unclear as to the role of SUB1785, but currently there is no evidence to link this to bacterial virulence.

The *S. uberis* serine protease SUB1154 is covalently anchored to the cell wall by SrtA and shares homology with the *S. pyogenes* ScpA protein. ScpA cleaves the C5a complement factor, inhibiting the recruitment and activation of phagocytic cells to the site of infection (Ward *et al*., 2009; Egan *et al*., 2012). Although such function has not been shown for SUB1154, the protein is required for colonisation and appears to prime the BMMO NLRP3 inflammasome by interacting with toll-interleukin receptor (TIR) domains within the TLR2 signalling pathway (Chapter 3). ScpA is under control of the *mga* regulon in *S. pyogenes*. Mga is similar to the positive transcriptional regulator, Vru (SUB0144) in *S. uberis* (McIver *et al*., 1995; Ward *et al*., 2009), however SUB1154 appears not to be under transcriptional control of SUB0144 in *S. uberis* (Egan *et al*., 2012).

Pathogenesis of *S. uberis* involves several virulence determinants that function to enable colonisation, persistence and virulence. Experimental exploitation of mutants lacking these proteins has suggested their contributions to *S. uberis* pathogenesis as they are required to establish intramammary infection and cause disease signs. However, the exact role of a number of these proteins has yet to be determined.

Table 1.1. *S. uberis* virulence determinants and their role during intramammary infection.

Gene*	Protein annotation*	Function	Role in *S. uberis* IMI
sub0881 (*srtA*)	Sortase A	Anchors a discrete subset of proteins to the cell surface (including SUB0145, SUB1095 and SUB1154).	High level colonisation. Signs of clinical disease.
sub0145 (*lbp*)	Lactoferrin binding protein	Binds lactoferrin. Biofilm formation. Facilitates internalisation into bMECs.	Binding lactoferrin is not essential for *S. uberis* colonisation. Evasion of the host immune system.
sub1635 (*sua*)	Putative membrane anchored protein	Binds lactoferrin. Facilitates adherence and internalisation into bMECs.	Binding lactoferrin is not essential for *S. uberis* colonisation. Evasion of the host immune system.
sub1095	Collagen-like surface anchored protein	Unknown in *S. uberis*. *S. pyogenes* SclB binds $\alpha_2\beta_1$ integrins expressed on fibroblasts, NK cell, Th1 cells and Th17 cells.	*S. uberis* colonisation. *S. pyogenes* evasion of the host immune system.
sub0144 (*vru*)	Putative Mga-like regulatory protein	Regulates expression of proteins (including SUB0145, SUB1095, SUB1785 and SUB1697).	Colonisation.
sub1697 (*hasA*)	Hyaluronan synthase	Hyaluronic acid capsule production. Facilitates adherence to bMECs.	Colonisation. Evasion of the host immune response (not resistant to the bactericidal effects of neutrophils).
sub1785 (*pauA*)	PauA protein precursor (streptokinase precursor)	Activates plasminogen to plasmin. Degrades host proteins into small peptides and amino acids.	Unknown (not for colonisation).
sub1154	C5a peptidase precursor	Primes the BMMO NLRP3 inflammasome by interacting with BMMO TIR domains†.	Colonisation. Activates the host immune system.

*Gene and protein annotation reference according to the NCBI protein database. †Chapter 3.
bMECs, bovine mammary epithelial cells; BMMO, bovine mammary macrophage; IMI, intramammary infection; TIR, toll-interleukin receptor; TLR2, toll-like receptor 2.

1.3.2. S. uberis interactions with neutrophils, macrophages and bMECs

Studies have found that *S. uberis* interacts with neutrophils, macrophages and bMECs differently compared to other intramammary bacterial pathogens. Tassi *et al*., 2013 and 2015, compared *S. uberis* strains associated with persistent (FSL Z1-048) and transient (FSL Z1-124) infections. This study showed that intramammary infusion with persistent infection strain caused clinical mastitis in all six challenged cows, whereas the transient strain did not cause any clinical signs and the bacteria were eliminated within 3 days. It was observed that the persistent strain was more resistant to macrophage killing, but less resistant to neutrophil killing compared to the transient strain. These data demonstrate that there are strain dependent differences that affect pathogenicity.

During *S. aureus* and *E. coli* intramammary infections, neutrophils are normally recruited to the mammary gland and constitute the highest percentage of the leukocyte population by 8h post challenge (Hill, 1981; Colditz & Watson, 1982). In contrast, studies have observed that the influx of neutrophils occurs at 24h post challenge with *S. uberis* in the bovine mammary gland (Egan *et al*., 2012). Additionally, levels of IL-8 were not detectable within milk from *S. uberis* infected mammary gland until 18h post challenge and these levels increased until 66h post challenge (Rambeaud, 2003). These data coincide with the delayed recruitment of neutrophils to the site of *S. uberis* infection within the mammary glands.

Additionally, *S. uberis* strains were found to be resistant to the bactericidal effects of neutrophils *in vitro*. It was thought that resistance to phagocytic killing of *S. uberis* was due to the expression of the hyaluronic capsule preventing opsonisation of antibodies. However, it was found that resistance was not mediated by inhibition of antibody binding as there was no difference in immunoglobulin G1 (IgG1), IgG2 or IgM binding between phagocytosis resistance and susceptible strains (Leigh & Field, 1994). Consistent with *in vitro* data, despite the large influx of neutrophils reported, there were a limited number of bacteria detected intracellularly within neutrophils. Also, the absence of the capsule did not alter the resistance of *S. uberis* to the bactericidal action of bovine neutrophils *in vivo* (Thomas *et al*., 1994; Field *et al*., 2003). Akin to *S. aureus* intramammary infections, *S. uberis* stimulates the production of NETs. However, these NETs were found to have a disruptive effect on bMECs and did not significantly inhibit *S. uberis* proliferation (Li *et al*., 2022). These data suggest that neutrophil killing of *S. uberis* is insufficient to control intramammary infection.

Bovine MECs are the first cells that detect *S. aureus* and *E. coli* intramammary infections through TLR2 and TLR4 respectively. Comparison of *S. uberis* infection in a murine model

showed that absence of TLR2 was coincident with increased tissue damage and significantly decreased bactericidal activities, such as the production of ROS. Therefore, suggesting the involvement of TLR2 in the recognition of *S. uberis* (Wan *et al*., 2020). In contrast, human embryonic kidney (HEK) 293 cells transfected with whole bovine TLR2 showed no response to *S. uberis*, whilst recognition of *S. aureus* was detected (Farhat *et al*., 2008). Responses from primary cultures of bMECs showed innate responses to *E. coli* and to a lesser extent against *S. aureus*, but no response was detected following challenge with *S. uberis* (Günther *et al.,* 2016b). Further experimentation using primary bMECs showed that *S. uberis,* both heat inactivated and live, did not induce a substantial innate immune response. However, isolated LTA from *S. uberis* was found to induce a strong immune response and activated the transcription factor for pro-inflammatory cytokines, NF-κB, in bMECs but not through the TLR2 pathway. This implies that LTA on *S. uberis* is inaccessible to receptors on bMECs likely because it is masked by other bacterial components or presented in a way that does not permit interaction with TLR2, explaining the lack of immune recognition (Günther *et al.,* 2016a). These data suggest that bMECs are unable to recognise *S. uberis* pathogens during intramammary infection, meaning bMECs do not initiate the immune response to *S. uberis*. Therefore, the immune response must be initiated through the recognition of *S. uberis* by other cells within the mammary gland, such as the resident macrophage population.

1.3.3. S. uberis interactions with the bovine mammary adaptive immune response

Production of IL-17A has also been demonstrated in bovine mammary glands during *S. uberis* infection. IL-17A was detected between 57-168h post challenge with *S. uberis*, which was found to coincide with the increase of CD4+ T-cells at 48-96h. Although not measured, it was suggested to be that the CD4+ T-cells were predominantly Th17 cells as these are the T-cell subset known to primarily secrete IL-17A (Tassi *et al.,* 2013), along with type 3 innate lymphoid cells (ILC3) (Rainard *et al*., 2020). It was also noted that there was an increase in IL-8 and TNF-α, along with several other pro-inflammatory cytokines (Tassi *et al.,* 2013). IL-17A and IFN-γ secretion was also found in cows immunised with *S. uberis* crude extract (Wedlock *et al*., 2014), further supporting the involvement of Th17 cells in the adaptive immune response to *S. uberis* intramammary infection.

There has been limited research into the CD8+ T-cell response to *S. uberis* intramammary infections. *In vitro*, CD8+ T-cells released IFN-γ and displayed substantial bactericidal activity toward *S. uberis* following exposure to *S. uberis* antigens (Wedlock *et al*., 2014). Interestingly,

of the CD8+ T-cells responding to *S. uberis* intramammary infection, a significant proportion were found to be memory T-cells (Denis *et al*., 2011).

There has been limited research into the adaptive immune response to *S. uberis* intramammary infections. It is largely assumed that *S. uberis* interacts with T-cells and B-cells in the same manner as other intramammary pathogens, however, due to the substantial differences observed regarding the innate immune system, further investigation is required.

Conclusion

S. uberis is the predominant cause of bovine mastitis in the UK and is largely treated using antibiotic therapy at the onset of clinical signs. Due to the rising concerns surrounding the overuse of antimicrobials and ultimately bacterial resistance to them, alternative therapies need to be researched. Exploitation of *S. uberis* mutants lacking identified virulence determinants has suggested their contributions to *S. uberis* pathogenesis. Investigating cellular responses to *S. uberis* has highlighted many differences in *S. uberis* intramammary infection compared to other mastitis causing pathogens such as *E. coli* and *S. aureus*. For example, bMECs are usually the first cells to detect the presence of bacteria within the mammary gland. However, studies have shown that bMECs are unable to recognise *S. uberis* (through TLR2) and the initiation of the immune response occurs via macrophages. Macrophages play a fundamental role in the innate immune response as they activate bMECs and other macrophages to secrete pro-inflammatory mediators that recruit neutrophils to the site of infection (albeit delayed and killing insufficient). Macrophages also branch the innate and adaptive immune response through antigen presentation to CD4+ T-cells. Therefore, further investigation into the interaction between mammary macrophages and *S. uberis* is essential to understand early disease pathogenesis and may offer opportunities to intervene and reduce disease.

1.4. Aims and objectives

Studies have suggested that it is bovine mammary macrophages (not bMECs) which constitute the initial interactions between the host and *S. uberis*. Recently presented data has indicated that the inflammasome is triggered in BMMOs when challenged with *S. uberis* and that this requires the presence of the *S. uberis* cell surface protein SUB1154 (Archer *et al*., 2020). Macrophages may then initiate the immune response through the secretion of cytokines that

interact with other resident leukocytes and/or bMECs, resulting in the recruitment of neutrophils to the site of infection.

Hypothesis: The *S. uberis* SUB1154 protein is essential for the production of IL-1β from bovine mammary macrophages via the NLRP3 inflammasome.

Aim: Characterise the communication between *Streptococcus uberis* and bovine mammary macrophages with respect to its role in disease pathogenesis.

Objective 1: Design a model to define the output of bovine mammary macrophages following interaction with *S. uberis*.

Objective 2: Define the interaction between *S. uberis* and bovine mammary macrophages with regard to the structure and function of the SUB1154 protein.

Objective 3: Determine differences in the bovine mammary macrophage response following interactions with different *S. uberis* strains.

Chapter 2: Establishing the model: *S. uberis* causes an inflammatory response in bovine mammary macrophages.

2.1. Introduction

Bovine milk contains a variety of cells including immune cells such as macrophages, neutrophils and lymphocytes, as well as a small number of epithelial cells, collectively referred to as somatic cells. The somatic cell count is a main indicator of milk quality (AHDB). The SCC may be elevated at the beginning and end of lactation but is most commonly associated with the recruitment of leukocytes into the mammary gland in response to infection (Souza *et al*., 2016). In the UK, a SCC of <100 cells/µL indicates an uninfected cow. Conversely, cows with a SCC of >200 cells/µL are classified as highly likely to have an intramammary infection (AHDB). According to the United States Department of Agriculture (USDA), the maximum bulk tank SCC level that can be used for dairy products in the UK, European Union, Australia, New Zealand and Canada is 400 cells/µL. In contrast, the United States legal maximum bulk tank SCC is 750 cells/µL and in Brazil, the SCC cannot exceed 1000 cells/µL (USDA, 2021). The somatic cell population is responsible for initiating the immune response to intramammary infections.

Bovine mammary epithelial cells initiate the innate immune response against most invading Gram-positive bacteria in the mammary gland. These cells detect the presence of bacteria, produce and release chemoattractant interleukins that rapidly recruit neutrophils to the site of infection (Rainard *et al*., 2022b). Neutrophils phagocytose the invading bacteria and induce apoptosis (Brinkmann *et al*., 2004; Roussel *et al*., 2017). Macrophages function in both the innate and adaptive immune responses. In conjunction with epithelial cells, macrophages can act innately through the phagocytosis of bacteria and secretion of interleukins (Grant & Finch, 1997; Denis *et al*., 2006). Additionally, macrophages play a role in the adaptive immune response by acting as antigen presenting cells to lymphocytes, including T-cells (Paape *et al*., 2002; Pidwill *et* al., 2021). However, studies have shown that the immune response in the mammary gland to *S. uberis* is different from other pathogens.

2.1.1. Bovine mammary gland immune response to S. uberis

Moyes *et al*., 2010, showed the responses from bMECs obtained during experimental intramammary infection were consistent with stimulation by activated macrophages rather than direct stimulation by bacteria. Consistent with this observation, Gunther *et al*., 2016a,

demonstrated *in vitro* that *S. uberis* failed to induce innate responses in primary bMECs, but did induce responses from macrophages derived from blood, an observation subsequently reproduced in macrophages obtained from bovine milk (Archer *et al*., 2020). There is also evidence of a delayed recruitment of neutrophils in response to *S. uberis* infection compared to other mammary pathogens. Typically, neutrophils are recruited within 8h, however, during *S. uberis* infection, cellular influx occurs 24h post challenge. *S. uberis* also possesses the ability to evade neutrophil bactericidal activities (Field *et al*., 2003). Due to the lack of involvement of bMECs and neutrophils against *S. uberis* intramammary infection, macrophages are considered a very important cell type involved in the initiation of the immune response.

2.1.2. Interactions between bovine mammary macrophages and S. uberis

The predominant leukocytes are macrophages, constituting 10-80% of the total cell population in a healthy mammary gland (Ostensson *et al*., 1988; Paape *et al*., 2002; Schwarz *et al*., 2011). Macrophages within the mammary gland can reside between epithelial cells in the mammary tissue, called ductal macrophages, or in the lumen within milk, known as bovine mammary macrophages (BMMOs) (Rainard *et al*., 2018b & 2022b). The majority of ductal macrophages are involved in the remodelling of the mammary gland during post-lactational involution, however, their dendritic like protrusions permit some level of gland lumen sampling for bacterial presence (Dawson *et al*., 2020). Due to the association of ductal macrophages with bMECs, it is most likely that BMMOs within the milk are the first to detect invading *S. uberis* through immune surveillance. This depends on random interactions within the milk and consequently the density of bacteria present dictates the frequency of contact and can determine the extent of the immune response (Rainard *et al*., 2018b).

The interaction between BMMOs and *S. uberis* was demonstrated to be not particularly efficient or effective (Denis *et al*., 2006). BMMOs from lactating cows were found to be able to kill a significant proportion of *S. aureus*. However, this was not the case with respect to *S. uberis*, which was found to multiply (two-fold increase in bacterial numbers at 2h post challenge) inside mammary macrophages meaning BMMOs failed to efficiently kill *S. uberis*. In contrast, mammary macrophages obtained during the dry period were shown to both phagocytose and kill *S. uberis*. Therefore, it was suggested that BMMOs have reduced effectiveness of phagocytic killing in lactating mammary glands, possibly due to the ingestion of milk components such as milk fat globules and casein micelles. In addition, BMMOs were found to be poor producers of the pro-inflammatory cytokine, interleukin-1 beta (IL-1β)

compared to blood monocytes and inefficient at presenting antigens to lymphocytes (Politis *et al*., 1991; Politis *et al*., 1992; Denis *et al*., 2006).

Despite the lack of ability for BMMOs to phagocytose invading *S. uberis*, they do initiate an immune response. Günther *et al*., 2016a, suggested that *S. uberis* stimulates macrophages via PRRs as macrophages challenged with *S. uberis* strongly activated NF-κB, which is mediated by PRR signalling. NF-κB is a transcription factor that upon activation translocates into the nucleus and upregulates transcription of immune genes, including IL-1β. Therefore, BMMOs stimulated by *S. uberis* produce and release IL-1β into the mammary gland where it interacts with other macrophages and bMECs (despite these cells not responding directly to the bacteria) to enhance the immune response and recruit neutrophils to the site of infection (Quinton *et al*., 2007; Archer *et al*., 2020). Challenge of isolated BMMOs with *S. uberis* strain 0140J increased concentration of IL-1β after 24h compared to no treatment (Archer *et al*., 2020). Therefore, the immune response from BMMOs can be measured by determining the production and secretion of IL-1β.

2.1.3. Utilising bovine macrophages experimentally

Previous studies have used both the murine mammary macrophage cell line RAW 246.7 (Ascanius *et al*., 2021) and bovine blood derived monocytes. Peripheral blood mononuclear cells (PBMCs) isolated from bovine blood samples were incubated in non-adherent Teflon bags for 7-8 days to allow monocyte differentiation into mature macrophages. Cell suspensions were then seeded into culture plates/dishes and macrophages were purified through selective adherence. As macrophages are the only adherent cells within the PBMC population, contaminating lymphocytes were washed away and the adherent macrophages remained (Burr *et al*., 2012; Menge *et al*., 2015; Gibson *et al*., 2016; Jensen *et al*., 2016).

Alternatively, monocytes were positively selected from isolated PBMCs using mouse anti-human CD14-coupled microbeads and magnetic activated cell sorting columns. CD14 (cluster of differentiation 14) is highly expressed on bovine monocytes and macrophages and so is commonly used to differentiate these cell populations (Hébert *et al*., 2000; Hussen & Schuberth, 2017). Purified monocytes were plated and differentiated into macrophages by the addition of 10 ng/mL recombinant bovine macrophage colony-stimulating factor (M-CSF) (Garcia-Sánchez *et al*., 2019; Ladero-Auñon *et al*., 2021). Although the use of blood monocytes is an improvement in comparison to RAW 246.7 cells as it avoids the issues that arise with immortalisation of cells and is of bovine origin, these cells have not undergone differentiation

into mammary specific macrophages. Therefore, these cells may act differently (Günther *et al*., 2016a).

This issue was overcome by extracting cells from raw bovine milk, generating an *ex vivo* model from the same population of cells normally present in the bovine mammary gland. Milk was diluted in either PBS (phosphate buffered saline), PAE buffer (PBS, acid-citrate dextrose, EDTA (ethylenediaminetetraacetic acid)) or PBS/EDTA/TE buffer; centrifuged; the fat layer and supernatant discarded and the pellet washed (Gibson *et al*., 2016; Silva *et al*., 2021; Archer *et al*., 2020). Several methods were used to subsequently isolate BMMOs from the somatic cell population. These included BMMO identification through fluorescence-activated cell sorting (FACS) with mouse anti-human CD14 antibodies (Silva *et al*., 2021; Hébert *et al*., 2000), selective adherence (Gibson *et al*., 2016) or mouse anti-human CD14 microbeads (Archer *et al*., 2020). Another method isolated BMMOs from milk diluted with MGS (modified Gey's balanced salt solution) and metrizamide (density gradient medium for centrifugation) and collected the bottom 10 mL after centrifugation in fractions of 1-3 mL by aspiration. Macrophages were identified by morphology and the yellow-reddish cytoplasm after acridine orange staining due to the high RNA content (Sandgren *et al*., 1991).

Isolation of BMMOs provides an accurate method to analyse specifically the response of these cells to *S. uberis*. However, the immune response is a complex pathway with multiple interactions that affect and intercept with each other and so may not be truly representative of the mammary gland immune response. Consequently, to understand the mammary gland immune response as a whole, determining specifically how macrophages function needs to be achieved. To assess the immune response between BMMOs and *S. uberis*, a replicable and reliable model for isolating BMMOs from milk, based on methods outlined in previous studies, needed to be determined and the parameters established by which these BMMOs are challenged with *S. uberis*.

2.2. Methods

2.2.1. Isolation of BMMOs from milk

Raw milk was collected from bulk tank at the University of Nottingham, Sutton Bonington campus dairy centre. The somatic cell count (SCC) was determined using a DeLaval Cell Counter (DCC). The somatic cell count (SCC) was determined using a DeLaval Cell Counter (DCC). Intramammary infection is often accompanied by an elevation in the SCC. Consequently, milk with a SCC >200 cells/µL was discarded as macrophages may have already been activated, which would directly affect subsequent results. Milk with a SCC of <200 cells/µL was processed to obtain the milk cell population.

Equal volumes of milk and PAE buffer (PBS + 10% acid-citrate dextrose (citric acid, Sigma, 33114; tris-sodium citrate, Fisher Chemical, S/P500/53; D-(+)-glucose, Sigma, G7528) + 20 mM EDTA (Thermo Scientific, J15694-AP) were centrifuged (40 min, 600xg) at 15°C, with acceleration 9 and deceleration 1 (Thermo Scientific, Megafuge^{TM} 16R). The pellet was resuspended and washed with PBS and cells obtained by centrifugation (10 min, 300xg) at 15°C, with acceleration 9 and deceleration 5. Cells were then washed twice in PBS with the addition of 2% antibiotic-antimycotic (Sigma, A5955) and 0.5 µg/mL Amphotericin B (Gibco, 15290018) (Harmsen *et al*., 2011) and cell density was measured (DCC). Finally, cells were resuspended in IMDM (Iscove's modified Dulbecco's medium, Gibco, 12440-046) containing L-glutamate and 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) + 10% foetal bovine serum (FBS, Sigma, F7524) + 2% antibiotic-antimycotic + 0.5 µg/mL Amphotericin B and plated at a density of $1x10^6$/well in a 24-well tissue culture plate and incubated overnight at 37°C and 5% CO_2.

Media was removed and each well was washed with PBS heated to 37°C to remove any nonadherent cells, leaving only the adhered BMMOs. Three wells were set aside for each plate where, following disposal of nonadherent cells, BMMOs from these wells were removed and the number of cells calculated using a haemocytometer (Marienfeld, 0640030) to verify the number of BMMOs in each well (~50,000). Cell viability was assessed by exclusion using 0.4% trypan blue solution (Sigma, T814).

2.2.2. Flow cytometry

A sample of isolated somatic cells and BMMOs were collected and washed three times by centrifugation at 300xg for 5 min and resuspended in FACS (fluorescence-activated cell sorting) buffer (0.5% Bovine Serum Albumin (BSA, Fisher Scientific, BP1605) in PBS). 1 µg PE-Cy7

(Phycoerythrin-Cyanine 7) mouse anti-human CD14 (cluster of differentiation 14, BD Pharmingen, 560919, clone M5E2) was incubated with 100 µL of each sample for 1h at room temperature in the dark. Washing was repeated followed by incubation in 4% paraformaldehyde (Sigma, 100496) for 15 min at room temperature. Samples were centrifuged and resuspended in ice cold PBS and stored at 4°C in the dark until analysed on a CytoFLEX S flow cytometer (Beckman Coulter).

2.2.3. Bacterial culturing conditions

S. uberis strain 0140J (strain ATCC BAA-854/0140J), originally isolated from a clinical case of bovine mastitis in the UK (ATCC® numbers BAA-854), was used throughout as a reference strain. *S. uberis* strain 0140J was cultured in Brain Heart Infusion (BHI) media (Oxoid, CM1135) at 37°C overnight. Bacterial cultures were washed 3 times in PBS at 5,000xg for 3 min and then heat-killed at 63°C for 30 min. Cultures were resuspended in IMDM at an optical density (OD) of 1 at 600 nm wavelength. The CFU/mL of *S. uberis* strain 0140J at an OD of 1 was determined by diluting the cultures 10-fold in saline and growing overnight at 37°C on THB agar plates (Todd-Hewitt Broth, Oxoid, CM0189 + 1.5% Agar Bacteriological, Oxoid, LP0011B).

2.2.4. Bacterial DNA extraction

S. uberis strain 0140J was cultured in BHI media at 37°C overnight. Cultures were centrifuged at 17,000xg for 10 min, resuspended in TE buffer (10 mM Tris (Sigma, 10708976001) and 5 mM EDTA) and centrifuged again at 17,000xg for 10 min. The pellets were resuspended in cell wall disruption buffer (TE buffer, 10 mg/mL lysozyme (Sigma, L7651) and 30 u/mL mutanolysin (Sigma, M9901)) and incubated for 30 min at 37°C. Lysis buffer (20% sodium dodecyl sulphate (SDS) w/v (Fisher Scientific, BP166) in 50 mM Tris, 20mM EDTA) containing 20 mg/mL Proteinase K+ (Sigma, P6556) was added and the tube inverted repeatedly until the suspension cleared, followed by incubation at 37°C for 1h. The protein and cell wall material were precipitated by the addition of 500 µL/mL saturated sodium chloride (6.0 M) and centrifugation at 17,000xg for 10 min. The pellet was discarded, and DNA was precipitated from the supernatant by addition of 1 mL 100% ethanol (stored at -20°C). DNA was collected by centrifugation at 17,000xg for 5 min and the pellet washed in 70% ethanol (stored at -20°C) followed by another centrifugation step at 17,000xg for 5 min. The final nucleic acid pellet was then air-dried and resuspended in TE buffer containing 20 µg/mL RNase A (Sigma, R4642) and incubated at room temperature for 30 min followed by 30 min at 37°C. The final DNA preparation was stored at 4°C (for use the following day) or -20°C (for longer term storage).

2.2.5. Polymerase Chain Reaction (PCR)

PCR of extracted bacterial DNA was completed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, M0530L) with primers (Sigma), described in Table 2.1 (10 µM in DNase free water). Manufacturer instructions were followed for 25 µL reactions with 2 µL of DNA per reaction with the PCR run on a thermocycler (Bioer Genetouch Thermal Cycler, TC-E-96GA). Thermocycling protocol described below in Table 2.2.

Table 2.1. DNA PCR primers.

Gene target*	Sequence	Function	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)
S. uberis sub0888	AM946015.1	Sortase anchored protein	CTTTATGAAAATAG CCAAGCTGAAA	TGTGAGCCAGAG GAGGAAG	60	974

*Gene according to the genomic sequence of *S. uberis* strain 0140J (Ward *et al*., 2009).

Table 2.2. DNA PCR thermocycling protocol.

Cycle Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	25 sec	30
Annealing	60°C	45 sec	
Extension	72°C	60 sec	
Final extension	72°C	5 min	1

2.2.6. Gel electrophoresis

Gel electrophoresis was conducted on the PCR products using a 1% agarose gel (Fisher Bioreagents, BP1356). PCR products were loaded onto the gel after being diluted in 6X HyPlus Loading Buffer Blue (Bioline, HYPB-214106) alongside a 1kB HyperLadder (Meridian Bioscience, HI-820109A). Gels were subjected to 100V for approximately 1h (Electrophoresis power supply, Consort, EV231; Analytikjena Biometra Compact M, 846-025-200) and then developed rocking in 0.5 µg/mL ethidium bromide solution (Thermo Scientific, 17898) before being imaged using a Biorad ChemiDoc™ Imaging System.

2.2.7. BMMO challenge

Following isolation, BMMOs were challenged with various stimuli as described in the associated narrative text (section 2.3) and IL-1β measured. The stimuli used were: either live or heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 1:1, 10:1, 50:1 or 100:1 bacterium:BMMO and lipopolysaccharide (LPS) (10 ng/mL; isolated from *E. coli* 0111:B4, Millipore, LPS25) (Xagorari *et al*., 2002) as a positive control.

2.2.8. ELISA

Bovine IL-1β was detected by ELISA using the Invitrogen Reagent Kit (ESS0027) following manufacturer's instructions. Coating antibody was diluted in BupH Carbonate/Bicarbonate Buffer (0.2 M, Invitrogen, 28382) and incubated overnight at room temperature in 96-well plates (Thermo Scientific, clear flat-bottom immune nonsterile, 3355). Plates were aspirated and incubated for 1h at room temperature in blocking buffer (4% BSA and 5% sucrose (Sigma, S0389) in PBS). Wells were washed with PBS + 0.05% Tween-20 (Sigma, P1379) and the detection antibody and streptavidin-HRP (horseradish peroxidase) were diluted in reagent diluent (4% BSA in PBS).

Absorbance was measured at wavelengths of 450nm and 550nm using a Varioskan® Flash multimode plate reader (Thermo Scientific). Optical imperfections were corrected for by subtracting the 550nm reading from the 450nm. A standard curve, using either second order polynomial (quadratic) or third order polynomial (cubic), was generated and sample IL-1β concentrations were interpolated.

2.2.9. Statistical analysis

Data was analysed using GraphPad Prism 10.0.3 (GraphPad Software, 2023). To determine if data was normally distributed a Shapiro-Wilk test was used. Normally distributed data was statistically analysed using either a 2-way ANOVA with Tukey's multiple comparisons post hoc test or an unpaired t-test. When data was found not to be normally distributed, a Kruskal-Wallis with Dunn's multiple comparisons post hoc test was used to determine significance. A value of $P \leq 0.05$ was considered to indicate a statistically significant difference. Spearman rank-order correlation coefficient was used to determine association, with nonlinear regression to calculate coefficient of determination.

Flow cytometry data was analysed using FlowJo v10.9.0. Initially gating was used to include all cells, followed by the exclusion of doublets and finally the percentage of CD14+ cells (Fig SI1).

2.3. Results

2.3.1. Cell numbers in whole bulk tank milk, isolated cells and seeded BMMOs

Initial attempts to isolate BMMOs using the technique employed by Archer *et al*., 2020, were unsuccessful. This was not improved using a different anti-bovine CD14 bead reagent kit (S-pluriBeads®, pluriSelect). Consequently, another more direct method was developed (2.2.1).

Designing and adapting the method for isolating BMMOs from milk was required to formulate a consistent and reliable model that could be utilised for further experimentations. Bulk tank milk SCCs were determined for each preparation to ensure they were within the guideline range and suitable for use (Table SI1). The SCCs were found to vary from 20 to 190 cells/µL, averaging 76 cells/µL ($2.25x10^8$ total cell count in 3L of bulk tank milk) with no statistically significant differences in SCCs seasonally (Fig 2.1A). Following the cell isolation protocol, the mean cell count was ~750 cells/µL ($3.5x10^7$ total isolated cells in 50 mL PBS suspension) (Fig 2.1B). A positive correlation was found between the SCC of whole bulk tank milk and the number of isolated cells obtained ($r = 0.51$) with a weak coefficient of determination ($R^2 = 0.15$) (Fig 2.1C).

Silva *et al*., 2021, found that there were ~6,000 BMMOs/mL of milk. In context, the total number of BMMOs in 3L of bulk tank milk would be $1.8x10^7$; 8% of the total SCC. Therefore, the predicted number of BMMOs in the isolated cell population would be $3x10^6$. Isolated cells were resuspended in media and seeded into culture dishes to contain approximately 100,000 BMMOs/well ($1x10^6$ isolated cell count/well). Following BMMO isolation through adherence, the actual macrophage cell numbers were calculated from all the experiments undertaken throughout the study and this showed a mean of 54,041 BMMOs/well (Fig 2.1D).

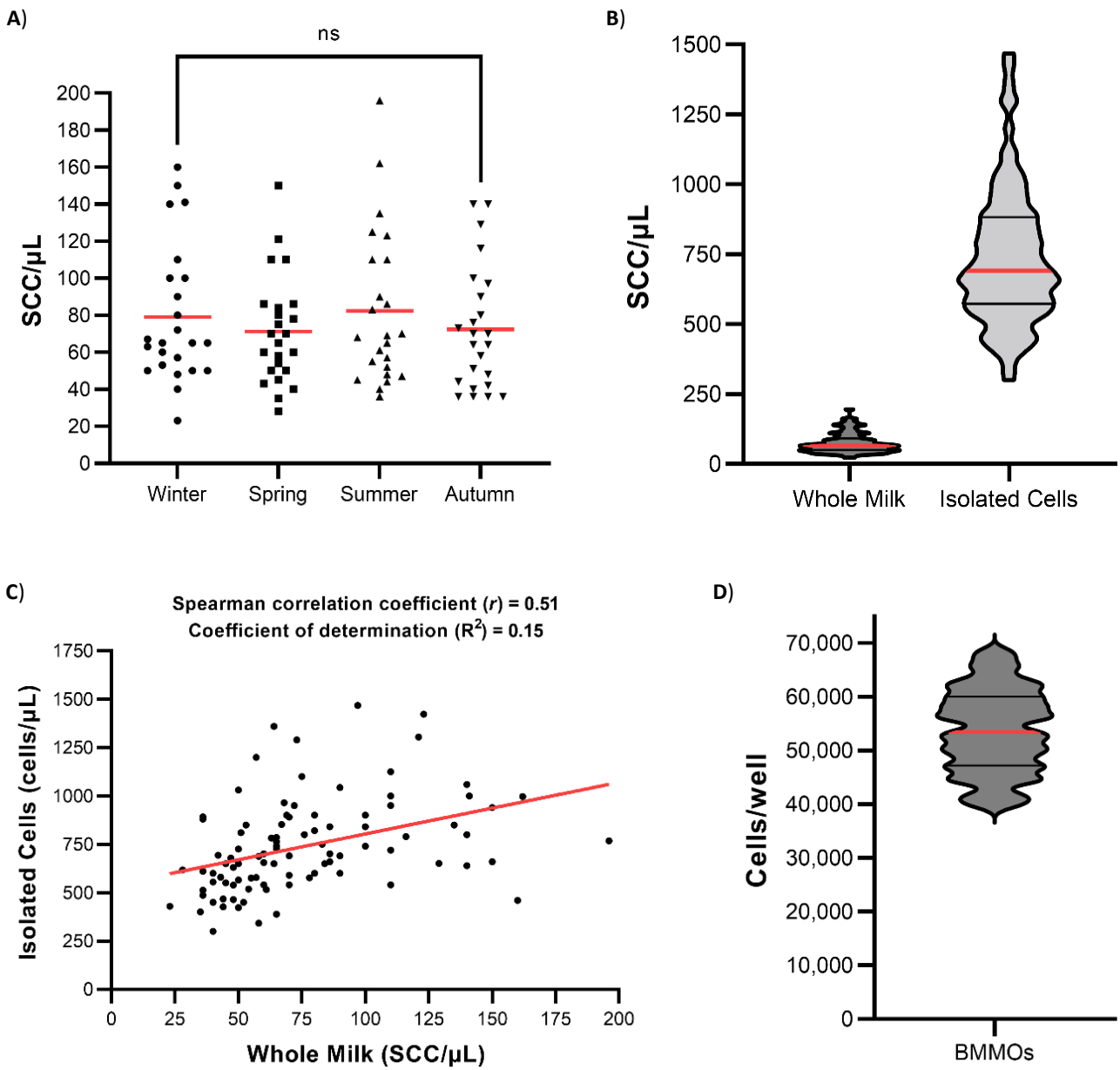


Fig 2.1. Cell numbers in whole bulk tank milk, isolated cells and seeded BMMOs. 3L of milk was collected from bulk tank between 2020-2023 and the somatic cell count (SCC) per µL was measured using a DeLaval cell counter. Milk was discarded if the SCC was >200 cells/µL. Graphs were determined using data provided in Table SI1. **A**) Milk SCCs were divided seasonally and presented as mean with N=24. Normality was determined by the Shapiro-Wilk test. Data was found to be not normally distributed and so was statistically analysed using a Kruskal-Wallis with Dunn's multiple comparison post hoc test. No significant differences (ns) were found between seasons. **B**) SCCs were determined in whole milk (3L) and following the cell isolation protocol, the isolated cell count in 50 mL PBS suspension. **D**) Number of isolated bovine mammary macrophages (BMMOs) were counted per well. Data (**B** and **D**) is presented as the medium, including upper and lower quartiles, with N=94. **C**) Association between the SCCs from whole milk and isolated cells were determined using Spearman rank-order correlation coefficient (positive correlation, r = 0.51). Nonlinear regression calculated the line of best fit and the coefficient of determination (R^2 = 0.15).

2.3.2. Percentage of CD14+ cells in the isolated cells and BMMO populations

The percentage of CD14+ cells within the isolated cells population was determined using flow cytometry analysis; CD14+ cells contributed 27% (Fig 2.2A). The percentage of CD14+ cells within the isolated BMMO population was measured at 63% (Fig 2.2B). Using trypan blue, the cell viability of the isolated BMMOs was >92%. Initially gating was used to include all cells, followed by the exclusion of doublets and finally the percentage of CD14+ cells. This data is also presented as heat maps for the isolated cells and BMMO populations (Fig SI1).

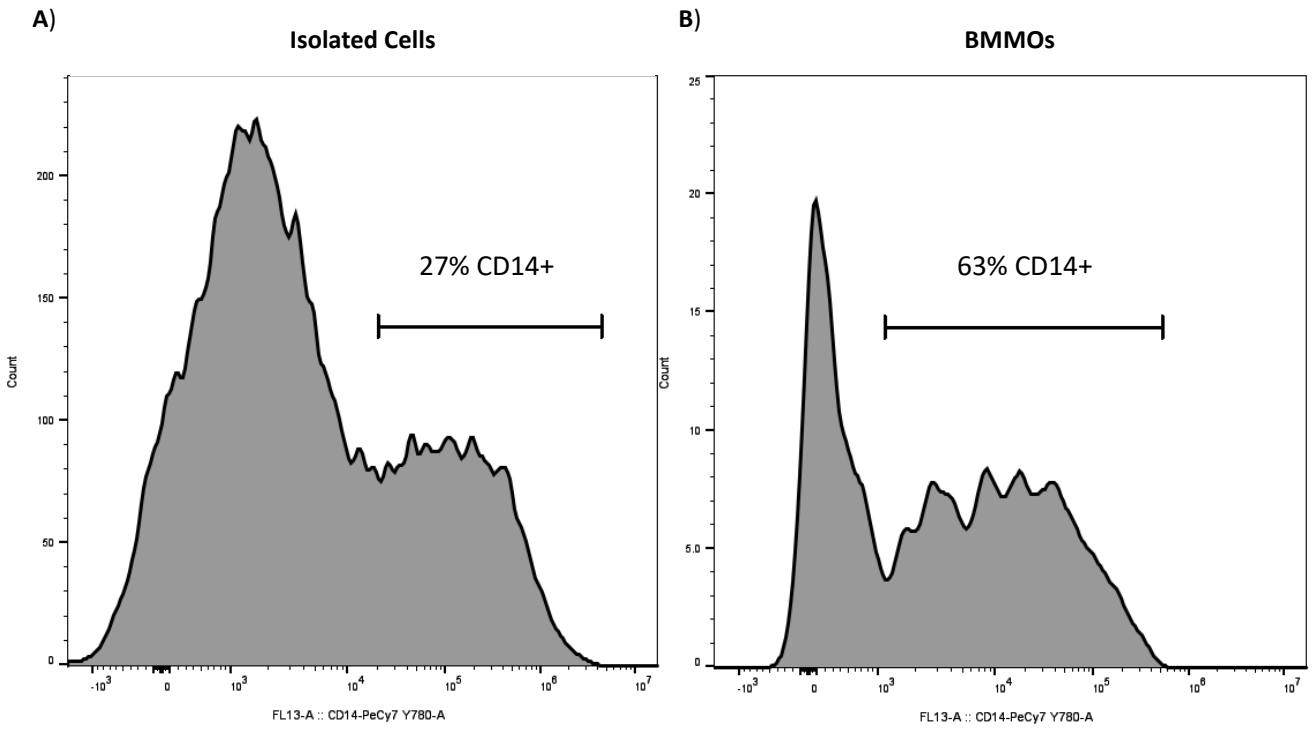


Fig 2.2. Percentage of CD14+ cells. Flow cytometry analysis was used to determine the percentage of CD14+ cells in the isolated cells (**A**) and bovine mammary macrophage (BMMO) (**B**) populations. The CD14+ population was determined using CD14-PeCy7 antibody. Initially gating was used to include all cells, followed by the exclusion of doublets and finally the percentage of CD14+ cells. This data is also presented as heat maps for the isolated cells and BMMO populations (Fig SI1).

2.3.3. IL-1β production from BMMOs over 24h following challenge with S. uberis strain 0140J

After establishing the method for isolating BMMOs, the specific parameters to evaluate the macrophage immune response to *S. uberis* needed to be determined to produce comparable data. Initially the optimal timepoint to evaluate the immune response was calculated. Isolated BMMOs were challenged with heat-killed *S. uberis* strain 0140J and the IL-1β concentration was measured every 2h over a 24h period (Fig 2.3). IL-1β production from BMMOs initially increased to ~45 pg/mL at 4h, which steadily decreased to ~7 pg/mL at 12h. IL-1β production from BMMOs then peaked at ~75 pg/mL at 20h followed by another decrease in IL-1β concentration to 24h. Due to the highest concentration of IL-1β measured at 20h, this was the focused timepoint in subsequent experiments.

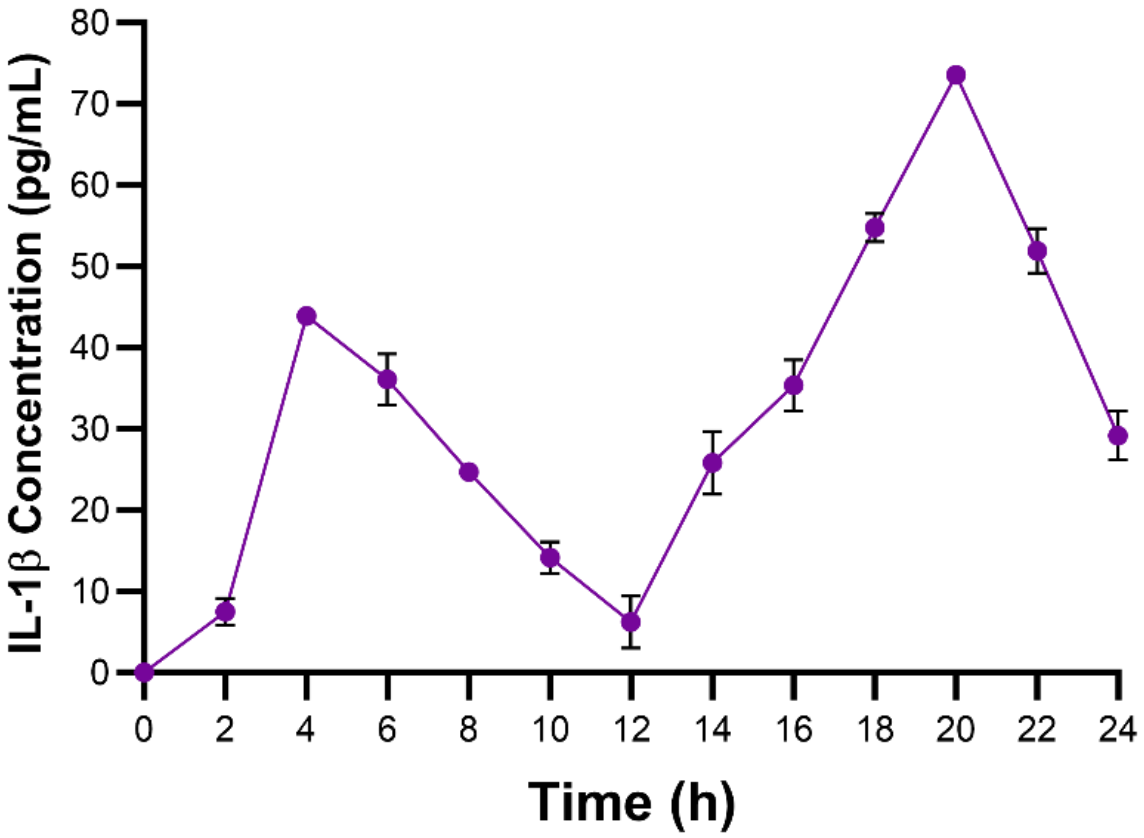


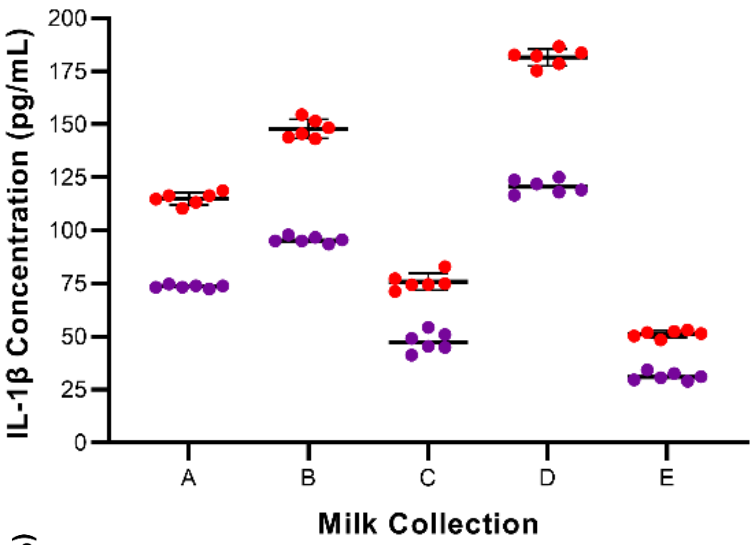
Fig 2.3. IL-1β production from BMMOs over 24h following challenge with *S. uberis* strain 0140J. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 50:1 0140J:BMMO. Supernatants were collected and the concentration of IL-1β was measured by ELISA every 2h over a 24h period. Data is presented as mean±SD with N=3 (technical and biological repeats).

2.3.4. Standardisation of IL-1β produced from BMMOs challenged with S. uberis strain 0140J to that obtained following BMMO stimulation with LPS

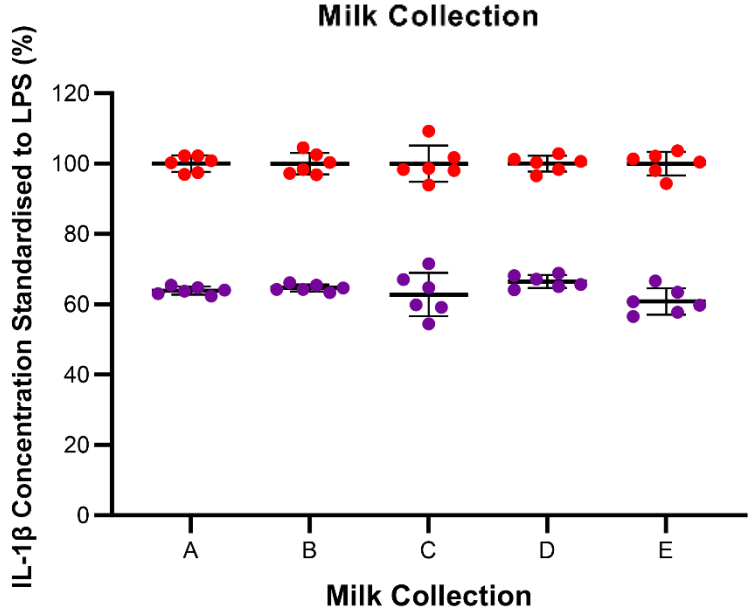
Stimulated BMMOs from each milk cell preparation were found to produce different concentrations of IL-1β. Comparability between datasets was determined by measuring the IL-1β produced from BMMOs challenged with *S. uberis* strain 0140J from five different bulk tank milk collections (Fig 2.4A) and standardising these values to the corresponding IL-1β concentration following LPS stimulation (Fig 2.4B). Standardisation was calculated by dividing the IL-1β concentration produced from BMMOs challenged with *S. uberis* strain 0140J to the mean IL-1β concentration obtained following LPS stimulation. These values were multiplied by 100 to determine the percentage of IL-1β concentration, with LPS at 100%. These data showed that despite the differing concentrations in IL-1β produced from BMMOs stimulated with LPS (51-182 pg/mL) and *S. uberis* strain 0140J (31-121 pg/mL), standardisation resulted in a consistent ratio of IL-1β produced (by LPS and *S. uberis* strain 0140J) between the different bulk tank cell preparations.

Challenge of BMMOs with *S. uberis* strain 0140J resulted in an IL-1β concentration at 60-64% of that obtained from LPS stimulation. For each bulk tank milk collection, the numbers of cells were recorded at each stage of the isolation protocol and the total cell numbers and isolated cells yield were calculated (Fig 2.4C). (Table SI1 for every milk collection). Milk collection D measured the highest: whole milk SCC/µL (110) and total SCC ($3.3x10^8$); isolated cells/µL (950) and total isolated cell count ($4.8x10^7$); IL-1β concentration from BMMOs stimulated with LPS and *S. uberis* strain 0140J and had the highest BMMOs/well (58,217). However, milk collection D had the lowest yield (15%) of isolated cells from whole milk. Conversely, milk collection E had the lowest: whole milk SCC/µL (28) and total SCC ($8.4x10^7$); IL-1β concentration and BMMOs/well (47,144) but had the highest yield (37%) of isolated cells from whole milk. Despite this, milk collection C had the lowest isolated cells/µL (539) with a total isolated cell number of $2.7x10^7$.

A)



B)



C)

Milk collection	Whole milk (SCC/µL)	Whole milk (Total SCC)	Isolated cells (cells/µL)	Isolated cells (Total cell number)	Isolated cells yield	BMMOs/well
A	50	$1.5x10^8$	725	$3.6x10^7$	24%	55,557
B	80	$2.4x10^8$	900	$4.5x10^7$	19%	49,120
C	48	$1.4x10^8$	539	$2.7x10^7$	19%	50,230
D	110	$3.3x10^8$	950	$4.8x10^7$	14%	58,217
E	28	$8.4x10^7$	617	$3.1x10^7$	37%	47,144

Fig 2.4. Raw IL-1β concentration vs IL-1β concentration standardised to LPS. Bovine mammary macrophages (BMMOs) were isolated from bulk tank milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 50:1 0140J:BMMO. Supernatants were collected 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were stimulated with LPS as a positive control (10 ng/mL). Raw IL-1β concentration was measured from 5 different bulk tank milk collections (**A**) and standardised to IL-1β produced from BMMOs stimulated with LPS in each corresponding milk collection (**B**). Standardisation was calculated by dividing the IL-1β concentration produced from BMMOs challenged with *S. uberis* strain 0140J to the mean IL-1β concentration obtained following LPS stimulation. These values were multiplied by 100 to determine the percentage of IL-1β concentration, with LPS at 100%. Data is presented as the mean±SD with N=3 (technical and biological repeats). **C**) For each milk collection, the somatic cell count (SCC) per µL was measured using a DeLaval cell counter on whole milk and cells/µL on obtained isolated cells. The total cell numbers were calculated in 3L milk and isolated cells in 50 mL PBS. The yield was calculated for the percentage of isolated cells obtained from whole milk. Following the BMMO isolation protocol, the numbers of macrophages/well were calculated using a haemocytometer.

2.3.5. Multiplicity of infection optimisation

The optimal multiplicity of infection (MOI) of *S. uberis* strain 0140J to BMMOs needed to be determined to accurately measure the immune response. Isolated BMMOs were challenged with varying MOIs of heat-killed *S. uberis* strain 0140J at 1:1, 10:1, 50:1 and 100:1. *S. uberis* strain 0140J:BMMO and the IL-1β concentration produced was measured by ELISA 20, 22 and 24h after challenge (Fig 2.5). Results were standardised to the LPS positive control at 20h. *S. uberis* strain 0140J caused BMMOs to produce 23%, 50%, 65% and 84% IL-1β at 1:1, 10:1, 50:1 and 100:1 respectively at 20h compared to that obtained from LPS stimulation. The concentration of IL-1β produced from BMMOs 20h after challenge with *S. uberis* at an MOI of 100:1 was significantly higher than that obtained at an MOI of 50:1 ($P<0.0001$), which, in turn, was significantly greater than that at an MOI of 10:1 ($P<0.001$). However, there was no significant differences in the concentration of IL-1β produced from BMMOs 22h after challenge with *S. uberis* at an MOI of 100:1, 50:1 nor 10:1. At 24h the concentration of IL-1β produced from BMMOs challenged with *S. uberis* was only significantly greater at an MOI of 100:1 compared to that obtained at 50:1 ($P<0.01$). Therefore, an MOI of 50:1 *S. uberis* strain 0140J:BMMO was determined as optimal in measuring IL-1β production from BMMOs 20h after challenge with *S. uberis*.

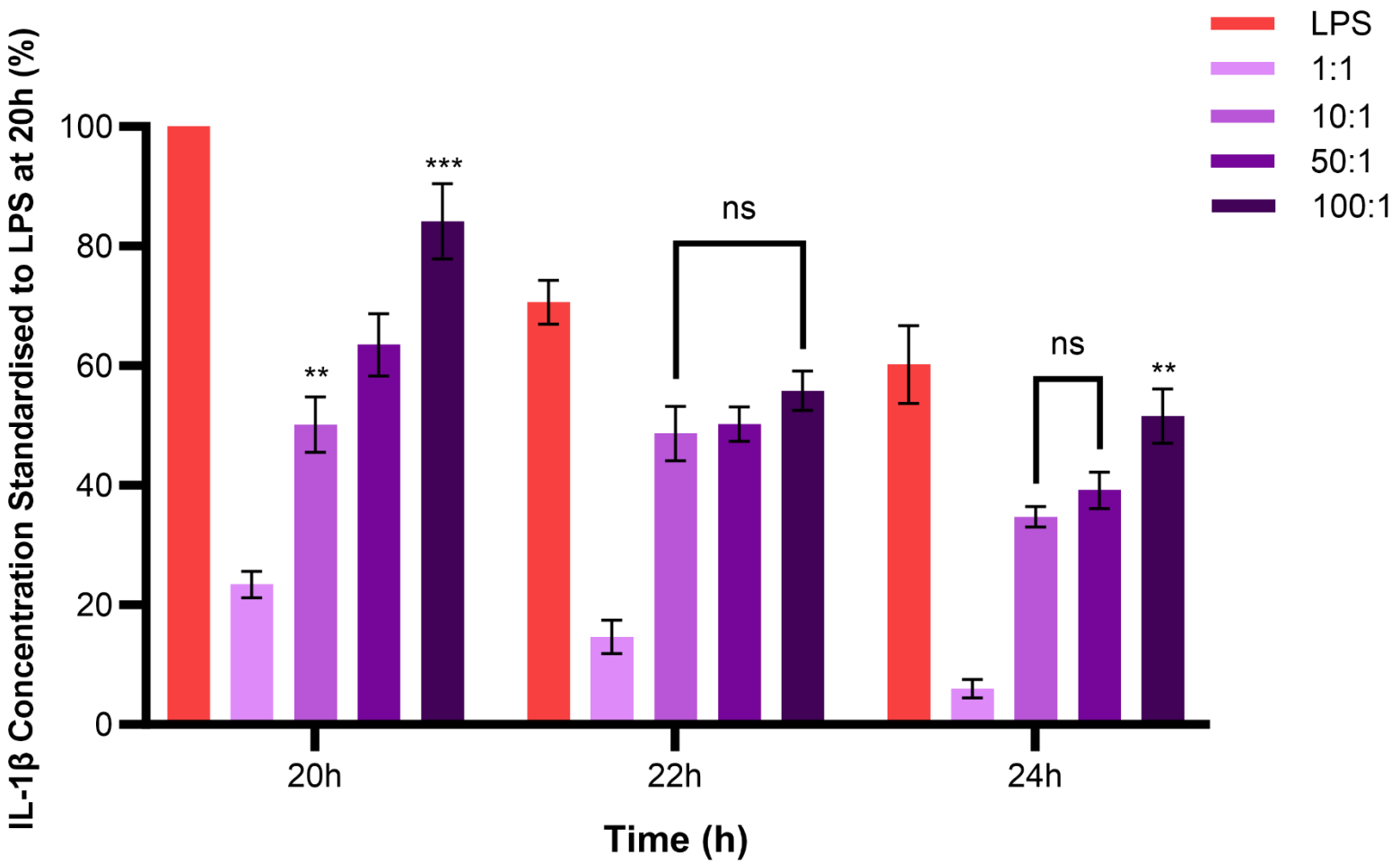


Fig 2.5. *S. uberis* MOI. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 1:1, 10:1, 50:1 and 100:1 *S. uberis* strain 0140J:BMMO. Supernatants were collected at 20, 22 and 24h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL) at 20h. Data is presented as mean of this ratio ±SD with N=6. Shapiro-Wilk determined the data to be normally distributed and so was statistically analysed using a two-way ANOVA with Tukey's multiple comparison post hoc test (***P<0.001 and **P<0.01 compared to 50:1 at the corresponding time).

2.3.6. Confirmation of S. uberis strain 0140J

Extracted DNA from cultures of *S. uberis* strain 0140J underwent PCR and gel electrophoresis. The PCR primers amplified SUB0888, encoding a sortase anchored protein, which is considered unique to *S. uberis* (Ward *et al*., 2009 and Sherwin *et al*., 2020). The gel image confirms that the bacterial cultures used throughout were *S. uberis* (Fig 2.6).

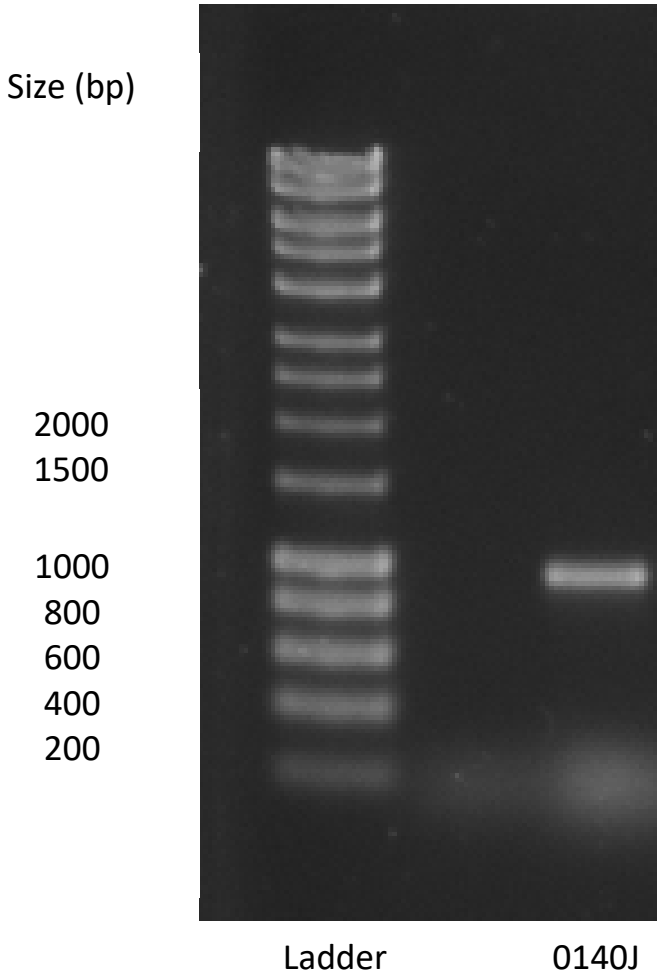


Fig 2.6. Confirmation of *S. uberis* strain 0140J. DNA was extracted from *S. uberis* strain 0140J culture, amplified by PCR using primers for sub0888 and imaged through gel electrophoresis. SUB0888 is considered unique to *S. uberis* and produces a product size of 974 base pairs (bp).

2.3.7. IL-1β production from BMMOs challenged with either live or heat-killed S. uberis strain 0140J

BMMOs were challenged with either live or heat-killed *S. uberis* strain 0140J and the concentration of IL-1β was measured 4h after challenge. *S. uberis* strain 0140J was heated for 30 min at 63°C (bacterial cells exposed to heating showed no growth on agar plates after 72h, whereas bacteria not heated above 37°C showed clear growth). The data showed that there was no statistically significant difference between IL-1β concentration produced by BMMOs stimulated with live or heat-killed *S. uberis* strain 0140J (Fig 2.7).

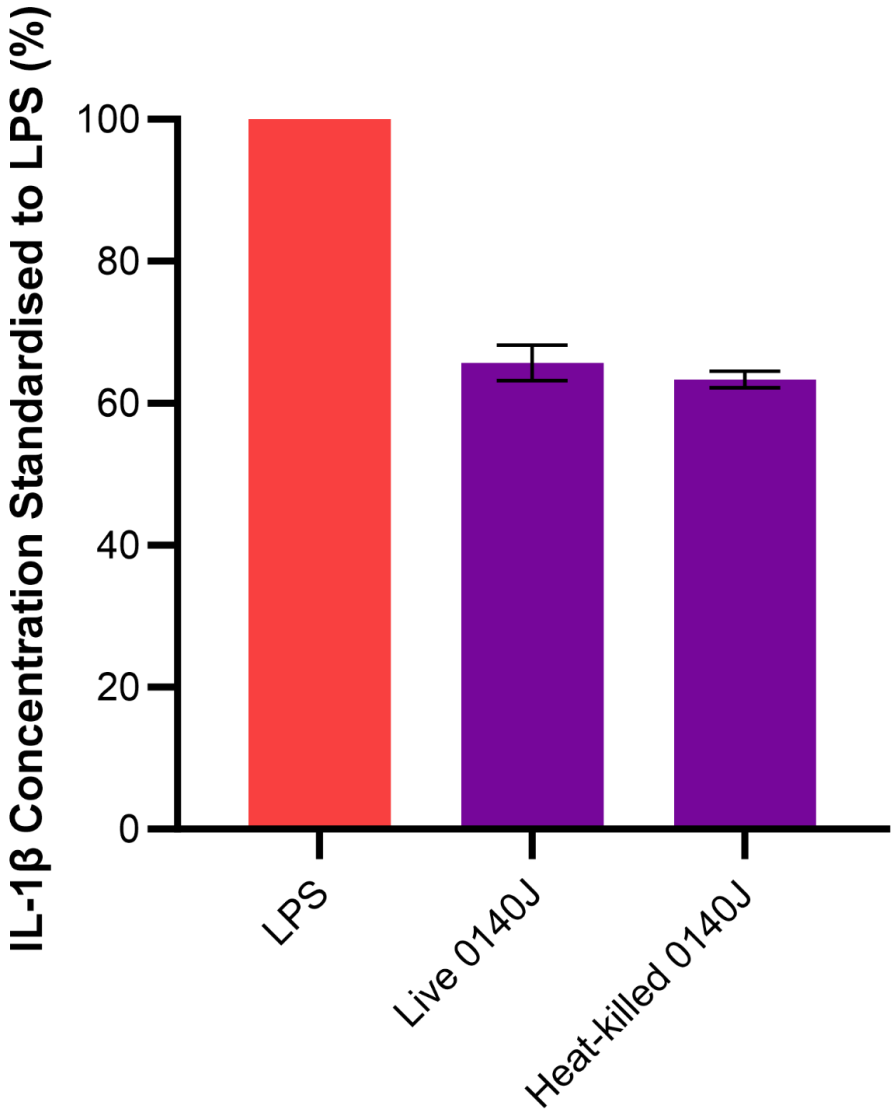


Fig 2.7. Live vs heat-killed *S. uberis* strain 0140J. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with live or heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 50:1 *S. uberis* strain 0140J:BMMO. Supernatants were collected 4h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as mean±SD with N=3 (assumed to be normally distributed as N too small) and statistically analysed using an unpaired t-test.

2.4. Discussion

2.4.1. Adaptations to the BMMO isolation method

The overall aim of this study was to investigate the initial interaction between BMMOs and *S. uberis* to further understand disease pathogenesis. To achieve this, a reliable model for isolating, challenging and measuring the response of BMMOs needed to be established. Initially, the protocol for isolation of cells outlined by Archer *et al*., 2020, was followed, however, adaptations were required to ensure more consistent and reliable isolation of cells.

2.4.1.1. Isolation of BMMOs by CD14 bead kits

Isolation of BMMOs using the human CD14 bead kit, adopted by Archer *et al*., 2020, was found to not be successful as it was discovered that the beads were no longer cross reactive with bovine samples potentially due to a change in antibody. To overcome this, anti-bovine CD14 S-pluriBeads® (pluriSelect) were tested, however, despite being bovine specific, there was a lack of binding to the beads with very low yields achieved during this study. These anti-bovine CD14 beads are no longer available as a product, perhaps due to the inconsistency of their performance. An alternative CD14 microbead kit was available that had been previously used by Garcia-Sánchez *et al*., 2019, and Ladero-Auñon *et al*., 2021, to isolate monocytes from bovine PBMCs, but, due to the lack of success with this method, this kit was not tested. Additionally, although some studies isolated BMMOs through FACS, these facilities were unavailable (Hébert *et al*., 2000; Silva *et al*., 2021). Consequently, BMMOs were routinely isolated from the milk leukocytes through adherence to plastic, as is well established (Fleit *et al*., 1984).

2.4.1.2. Isolation of BMMOs through adherence

The final protocol developed in this chapter exploited the fact that macrophages readily stick to plastic (and glass) while other leukocytes typically do not. Initially, the somatic cells were incubated in a tissue culture flask and the non-adherent cells discarded. The adhered BMMOs were removed using ice-cold PBS for minimal cell damage and transferred into wells in culture dishes. However, reduction of subsequent adherence and activity of BMMOs was evident using this method; this issue was also reported by Fleit *et al*., 1984. Therefore, isolated cells were directly added to culture dishes to retain BMMO adherence and activity. In accordance with the literature, isolated cells were incubated for varying times (3-18h) to allow the BMMOs to adhere, with the longer time period deemed optimal (Burr *et al*., 2012; Menge *et al*., 2015; Gibson *et* al., 2016; Jensen *et al*., 2016). The number of isolated cells seeded into culture dishes

varied from $8x10^4$-$1x10^6$ cells/well (Gibson *et al*., 2016). Therefore, $1x10^6$ cells in total were seeded meaning, after washing with PBS to remove contaminating leukocytes, approximately a maximum of $8x10^4$ BMMOs/well would remain. The limitation with this method resulted in less control over the number of BMMOs seeded into each well. For each cell preparation, wells were left aside and the BMMOs from these were removed; cell numbers were calculated to ensure there were approximately 50,000 BMMOs in each well. This was further corrected for in the assays by standardisation to LPS.

2.4.1.3. Utilising FACS to determine the purity of the isolated BMMOs

Bovine milk contains a complex cell population consisting of several different cell types including macrophages, neutrophils and lymphocytes. The proportion of BMMOs within milk varies within the literature from 10-80% of the total cell population in a healthy mammary gland (Ostensson *et al*., 1988; Paape *et al*., 2002; Schwarz *et al*., 2011; Alnakip *et al*., 2014). CD14 is a glycoprotein expressed on monocytes, macrophages, and to a lesser extent neutrophils, and is involved in receptor binding to invading pathogens as part of the innate immune response (Antal-Szalmas *et al*., 1997). The prevalence of CD14 is well established in mouse and human models, however it is not well characterised in BMMOs. FACS analysis found a clear subset, 27%, of the isolated cells from milk were CD14+.

Macrophages can be separated from complex cell populations due to their ability to adhere to plastic (Fleit *et al*., 1984), which was evident by the population becoming simpler following this method. Several studies have also implemented adherence as a method for isolating macrophages (Burr *et al*., 2012; Menge *et al*., 2015; Gibson *et al*., 2016; Jensen *et al*., 2016). FACS analysis of this population found 63% were CD14+. However, despite a high proportion of cells reported as CD14-, this does not mean they are not macrophages. CD14 expression can increase and decrease over time, with a proportion of macrophages being CD14- (Sladek & Rysanek, 2008 & 2014; Lambert *et al*., 2017). Chemicals such as trypsin and accutase have the potential at removing cell markers, including CD14 (Lai *et al*., 2022), therefore, BMMOs were removed from culture dishes using ice cold PBS. Vigorous washing with PBS is required to remove macrophages from plastic and so this may result in damage to the cells. This could mean some of the cells observed were fragmentated, reducing the representation of CD14 on these cells. Alternatively, other fragments and debris from milk may be misrepresented as cells, thereby reducing the percentage of CD14+ cells further. Additionally, there is a lack of available bovine specific antibodies and therefore, the human anti-CD14 antibody used may not bind

efficiently resulting in an under representation of the CD14+ cell population. Together, these data indicate that adherence to plastic was a successful method for isolating BMMOs.

The threshold for determining CD14+ cells was found to be lower for isolated BMMOs compared to isolated cells from milk. This could be due to the change in cell size as isolated cells from milk were exposed to a different environment with a variety of cells to interact with (Lee *et al*., 2013; McWhorter *et al*., 2013). In comparison, isolated BMMOs were removed from the other somatic cells and incubated overnight in culture medium. This change in environment could also contribute to a change in CD14 expression. Consequently, isolating macrophages through adherence may generate a different cell population to that used by Archer *et al*., 2020, as the cells used in that study were all CD14+. Therefore, some conformations of the earlier observations were required prior to advancing the investigation.

2.4.1.4. Low yield of isolated BMMOs

Isolation of cells from milk resulted in a large loss in cell number with an average yield of 19%. This could have been due to the general loss of cells through multiple centrifugations. Milk contains a high concentration of fat (4-5%), which accumulated as a layer at the top of the suspension after centrifugation. Cells could have become confined within this layer, which was removed, contributing to the reduction in yield. These issues would also have occurred using the bead separation method, so were inevitable. Another step that could explain the reduction in cell number was the adherence of BMMOs to the culture dishes. As the whole isolated cell population needed to be seeded, although other cells would not adhere (Burr *et al*., 2012; Menge *et al*., 2015; Jensen *et al*., 2016), they may have settled on the well resulting in the BMMOs not being in direct contact with the plastic to adhere. Therefore, some BMMOs would have been removed along with the other non-adherent cells during the PBS wash stages. This was accounted for as different sized culture dishes were trialled, with the 24-well dish generating the highest yield.

The final method outlined in this chapter was used throughout the study and proved to be reliable and reproducible.

2.4.2. Parameters of the BMMO isolation method

After the model had been developed, the specific parameters that were to be kept consistent throughout needed to be determined in order for the data to be comparable.

2.4.2.1. Standardisation of IL-1β concentration to LPS

It was found that BMMOs from different milk collections produced different concentrations of IL-1β following stimulation with *S. uberis* strain 0140J. This resulted in issues comparing data between cell preparations. Therefore, the IL-1β concentration produced from BMMOs for each cell preparation challenged with *S. uberis* strain 0140J were standardised to that obtained following stimulation with a standard dose of LPS. This method of standardisation produced very consistent data; challenge with *S. uberis* strain 0140J produced 62% of that obtained from LPS stimulation. This indicated that although each preparation of BMMOs produced different concentrations of IL-1β, the consistent ratio between IL-1β concentration after LPS and *S. uberis* strain 0140J suggested this related to the quality and possibly quantity of cells used. Removing the sample variation through this standardisation allowed for data to be compared between cell preparations, including the utility of the methodology.

The isolated BMMO population from bulk tank milk contains macrophages from different cows and therefore express varying MHCI molecules. Inadvertent interactions between BMMOs expressing varying MHCI molecules was eliminated by measuring the production of IL-1β from unstimulated BMMOs which was subsequently deducted from the other values.

2.4.2.2. Differing numbers of BMMOs

Wells were seeded with $1x10^6$ isolated milk cells in an attempt to obtain ~50,000 BMMOs for investigation; the final number of BMMOs differed. This could explain why cells obtained from milk collection D had the highest production of IL-1β following challenge with *S. uberis* as this preparation had the highest number of BMMOs (58,217/well). Similarly, cells obtained from milk sample E, which had the lowest number of BMMOs (47,144/well) showed the lowest production of IL-1β after challenge with *S. uberis*. However, a simple relationship between cell number and production of IL-1β was not the case, as cells from milk sample A produced lower levels of IL-1β than those from sample B despite a greater number of BMMOs (55,557/well compared to 49,170/well).

2.4.2.3. IL-1β production from BMMOs following challenge with S. uberis strain 0140J

Archer *et al*., 2020, measured the IL-1β concentration 24h after challenge to evaluate the immune response. To determine the optimal time to measure the IL-1β concentration, BMMOs were challenged with *S. uberis* strain 0140J over a 24h period and the IL-1β concentration was measured every 2h. Two peaks in IL-1β concentration were observed at 4h and 20h, with the latter being the greatest. Macrophages can detect pathogens through a variety of methods.

For example, macrophages sample the extracellular environment during immune surveillance, which can result in the uptake of an invading pathogens (Paape *et al*., 2000). The presence of pathogens can also be detected by binding of PAMPs to PRRs expressed by macrophages either extracellularly or intracellularly. These PRRs include TLRs (Toll-like receptors), NLRs (NOD-like receptors), intracellular RNA or DNA receptors (RIG-1 like receptors (RLR) or STING (stimulator of interferon genes) respectively), C-type lectin receptors and scavenger receptors (Ley *et al*., 2016). Alternatively, pathogens can become opsonised by host antibodies (IgG) and/or complement components (C3b), which bind to their respective receptors expressed on macrophages to facilitate phagocytosis (Acharya *et* al., 2020). This results in downstream signalling pathways that increase pro-IL-1β transcription, which is cleaved by the activated NLRP3 inflammasome, ultimately resulting in the release of IL-1β in its active, pro-inflammatory form (Chen *et al*., 2017 and Kaneko *et al*., 2019). Macrophages also express cytokine receptors that amplify the macrophage response. Therefore, the binding of secreted IL-1β to IL-1 receptors on neighbouring cells would create a positive feedback loop activating more macrophages resulting in production of further IL-1β. Hence, my interpretation of these data is that the first peak most likely represents the initial secretion of IL-1β in response to the pathogen and the second peak is the enhanced response to the pathogen and release of IL-1β (Ley *et* al., 2016; Kaneko *et al*., 2019). In subsequent studies, IL-1β concentration was measured at 20h as this was identified as the maximal response and allowed for more reliable measurement of IL-1β concentration.

2.4.2.4. Optimal multiplicity of infection

As well as the optimal time to measure the BMMO immune response to *S. uberis*, the optimal MOI was determined. An MOI of 50:1 was selected for subsequent analysis as this challenge dose produced a reproducible response of 65% of that obtained with a standard dose of LPS. This allowed for reliable measurement of both greater and lower production of IL-1β within the range of the positive control condition (challenge with LPS) during the subsequent investigation. Challenge with MOIs 10:1, 50:1 and 100:1 resulted in significantly different levels of IL-1β at 20h after challenge, further indicating that the measurement of IL-1β concentration using an MOI of 50:1 at 20h post challenge was optimal to investigate changes (higher and lower) in the production of IL-1β.

2.4.2.5. IL-1β production from BMMOs following challenge with live or heat-killed S. uberis

To accurately control the *S. uberis* strain 0140J:BMMO MOI throughout the 20h after challenge, IL-1β concentration was measured following challenge with either live or heat-killed *S. uberis*

strain 0140J. No significant difference was found, implying that this reaction was not dependent on or enhanced by factors secreted by live bacterial cells. Heat-killed *S. uberis* was therefore used in following experiments. Although this does not represent *in vivo* conditions, it removes the variability of bacterial number that may occur due to bacterial growth and permitted experiments to be conducted in the presence of antimicrobials allowing comparisons between datasets and analysis of the BMMO immune response. Similarly, Günther *et al*., 2016a, reported that there was no difference between live or heat-killed *S. uberis* in inducing expression of immune genes in bMECs.

Conclusion

A method based on cell isolation and adherence to plastic was established to isolate a yield of approximately $1.8x10^6$ BMMOs from 3L of raw bovine milk. Seeding of approximately 50,000 BMMOs/well into each compartment of a 24-well culture dish produced reliable and reproducible data with regard to production of IL-1β at 20h in response to challenge with *S. uberis* at an MOI of 50:1 compared to a standard dose of LPS.

Chapter 3: *S. uberis* SUB1154 protein primes the BMMO inflammatory response

3.1. Introduction

3.1.1. S. uberis SUB1154 protein

S. uberis initiates the immune response in BMMOs through stimulating the production of the pro-inflammatory cytokine IL-1β (Chapter 2). Recent studies have shown that colonisation and virulence are substantially reduced by the deletion of *sub1154* in *S. uberis* strain 0140J (Archer *et al*., 2020). The gene encodes a sortase anchored cell envelope serine protease, that has previously been labelled a putative C5a peptidase precursor due to its homology (34%) with ScpA in *Streptococcus pyogenes* (Ward *et al*., 2009). Although ScpA in group A streptococci has been shown to cleave the C5a complement factor, reducing the recruitment and activation of phagocytic cells to the site of infection (Ji *et al*., 1996), no such function has been shown regarding *S. uberis* SUB1154. *S. uberis* fails to induce innate responses directly from bMECs, suggesting it is BMMOs that initiate the initial host immune response (Moyes *et al*., 2010; Günther *et* al., 2016a). Therefore, how SUB1154 is able to elicit IL-1β production via the inflammasomal pathway needed to be understood in order to determine the function of SUB1154 in the BMMO immune response.

3.1.2. Inflammasomal complexes

Stimulation of macrophages by bacteria results in the initiation of a signalling cascade that leads to the formation of the inflammasome and expression of inactive pro-inflammatory cytokines such as pro-IL-1β and pro-IL-18 (Sharma & Kanneganti, 2016). Several inflammasome complexes have been characterised including NLRP1, NLRP3, NLRC4, pyrin and AIM2, with evidence emerging of further complexes such as NLRP6, NLRP7 and IFI16 (Guo *et al*., 2015). Each inflammasome complex differs in the cells it is expressed in and what activators cause assembly (Fig 3.1).

Bacterial toxins are important inflammasome assembly activators of NLRP1 and pyrin. NLRP1 is mainly expressed in motor neurons, microglia and epithelial cells with assembly either directly activated by lethal toxins from bacteria including *Bacillus anthracis* and *Shigella flexneri* or indirectly activated by *Toxoplasma gondii* (Broz & Dixit, 2016; Mi *et al*., 2022). Pyrin is confined to innate immune cells including granulocytes, eosinophils, monocytes and

dendritic cells and detects bacterial toxin-induced Rho GTPase inactivation, such as *Clostridium difficile* toxin B and *Clostridium botulinum* C3 toxin (Xu *et al*., 2014; Schnappauf *et al*., 2019).

Macrophages express a variety of inflammasome complexes including NLRP3, NLRP7, NLRC4 and AIM2. NLRC4 assembly occurs in response to bacterial flagellin or rod and needle subunits of type 3 or 4 secretion systems, such as those found in *Salmonella* (Duncan & Canna, 2018; Schnappauf *et al*., 2019). AIM2 directly binds to cytosolic dsDNA from a variety of pathogens (viruses, bacteria following lysis, fungi, and protozoa) as well as self-DNA (Broz & Dixit, 2016; Kumari *et al*., 2020). NLRP7 is not well characterised but has been shown to assemble following activation from microbial lipopeptides, including those from Gram-positive bacteria, *Mycoplasma spp.* and *Mycobacterium bovis* (Khare *et al*., 2012; Schnappauf *et al*., 2019).

Some inflammasome complexes have been found to operate in more niche and specific locations. NLRP6 is highly expressed in intestinal epithelial cells (enterocytes), T-cells, monocytes and neutrophils where it plays an important role in controlling intestinal homeostasis by protecting against infections and functions in the production of intestinal mucus and glucose regulation. NLRP6 has also been found to have roles in other organs and tissues including the joints, lungs, oral cavity, liver, kidney and nervous system (Angosto-Bazarra *et al*., 2022). IFI16 is specifically expressed in the nuclei of endothelial cells and is directly associated with IFN-β responses to viral and bacterial DNA (Kerur *et al*., 2011).

NLRP3 is the most well studied inflammasome complex and has been shown to assemble following activation from a variety of different stimuli, some of which include crystalline and particulate matter (such as uric acid crystals, silica, asbestos, and alum), extracellular ATP, pore-forming toxins, RNA, DNA, and several viral, bacterial, fungal, and protozoan pathogens (Broz & Dixit, 2016). Previous studies have shown that *S. uberis* inflammasome activation is via the oligomerisation of NLRP3 and the action of caspase-1 to elicit IL-1β release from mouse MECs (Yan *et al*., 2022) and BMMOs in a SUB1154-dependent manner (Archer *et al*., 2020). This has led to a working hypothesis in which priming occurs in the absence of SUB1154, which subsequently activates the inflammasome (Archer *et al*., 2020). Therefore, the precise mechanism by which *S. uberis* and SUB1154 results in the production of inflammatory cytokines in BMMOs in an NLRP3-depenedent manner needed further elucidation.

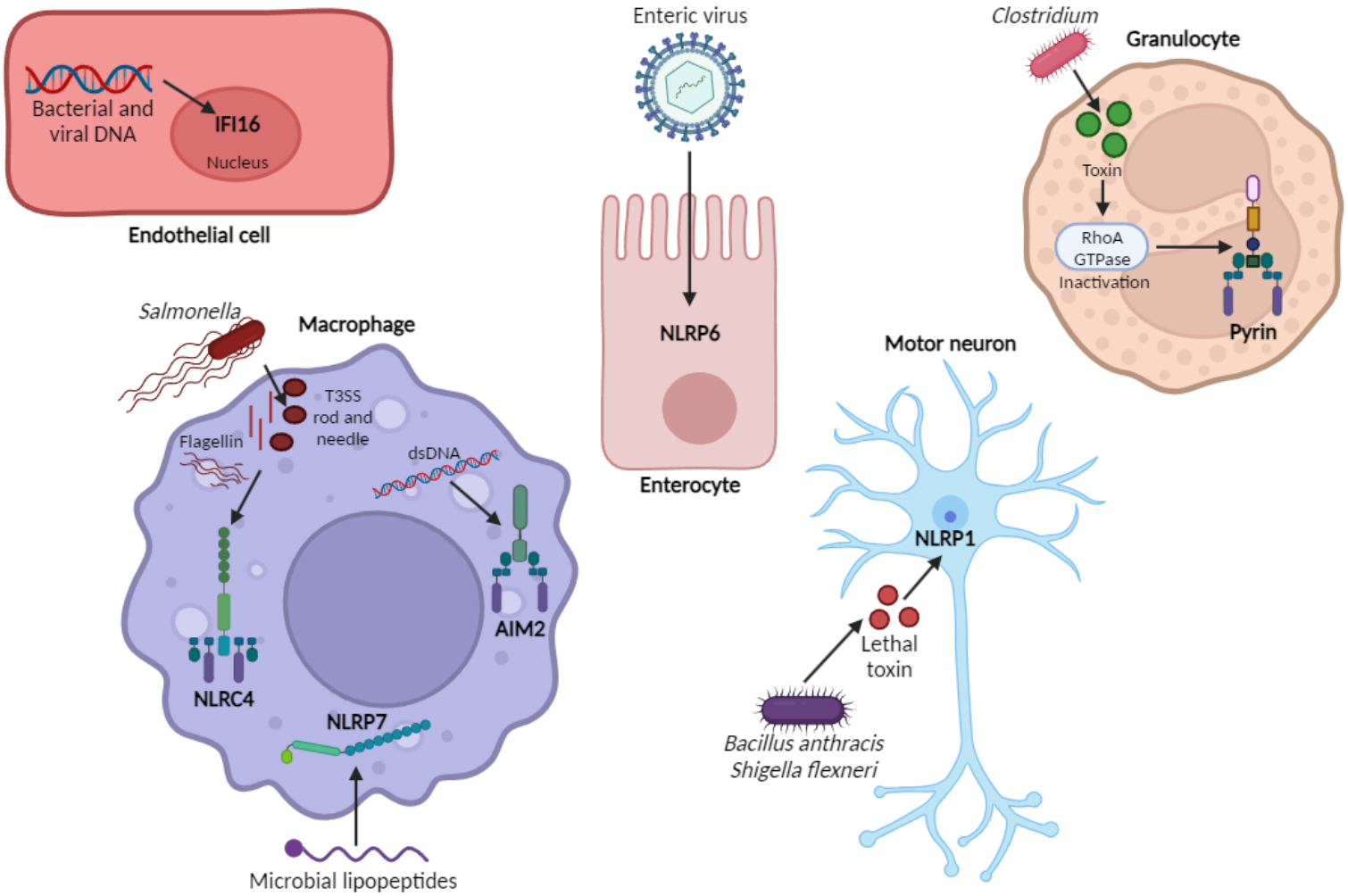


Fig 3.1. Types of inflammasomal complexes. NLRP1 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-1) is mainly expressed in motor neurons and is directly activated by lethal toxins from bacteria including Bacillus anthracis and Shigella flexneri. NLRP6 is highly expressed in intestinal epithelial cells (enterocytes) and can be activated by a variety of pathogens, for example, enteric viruses. Macrophages express a variety of inflammasomal complexes including NLRP7, which assembles following activation from microbial lipopeptides; NLRC4 (NLR family caspase activation and recruitment domain containing 4), which is activated by flagellin or rod and needle subunits of type three secretion system (T3SS) from bacteria such as Salmonella and AIM2 (absent in melanoma 2), which directly binds cytosolic double stranded DNA (from pathogens or self). Pyrin is confined to immune cells such as granulocytes (neutrophils and eosinophils) and is activated by Clostridium bacterial toxins which inactive RhoA GTPase. IFI16 is specifically expressed in the nuclei of endothelial cells and associates with bacterial and viral DNA. Figure created using BioRender.com.

3.1.3. NLRP3 inflammasome structure and assembly

Assembly of the NLRP3 inflammasome is a complex, multifactorial process used by innate immune cells, most commonly macrophages, to secrete pro-inflammatory cytokines to promote an immune response to invading pathogens. NLRP3 inflammasome is a supramolecular organising centre consisting of a sensor (NLRP3 protein), adaptor (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC) and an effector (caspase-1) (Mamantopoulos *et al*., 2017; Swanson *et al*., 2019; Blevins *et* al., 2022). The NLRP3 tripartite protein contains a C-terminal leucine rich repeat (LRR), ATPase-containing NACHT (NLRP3 nucleotide-binding and oligomerisation) domain that mediates oligomerisation and an N-terminal pyrin (PYD) domain involved in protein recruitment for inflammasome assembly (Fig 3.2) (Kelley *et al*., 2019; Blevins *et al*., 2022). NLRP3 oligomerises into a circular cage structure and recruits ASC, via PYD-PYD polymerisation, as helical filaments that nucleate forming ASC macromolecular specks that subsequently recruit pro-caspase-1 via the caspase activation and recruitment domain (CARD). NLRP3 oligomerisation is mediated by NEK7 (never in mitosis gene A (NIMA)-related kinase 7) which binds to the LRR and NACHT domain of NLRP3 and connects NLRP3 subunits together into a complex that is required for ASC speck formation and caspase-1 activity (Shi *et al*., 2016; Sharif *et* al., 2019; Andreeva *et al*., 2021; Hochheiser *et al*., 2022). This allows for pro-caspase-1 self-cleavage into two domains, one of which is proteolytically able to cleave pro-IL-1β and pro-IL-18 to active cytokines that are secreted out of the macrophage to initiate an immune response (Manji *et al*., 2002; Martinon *et al*., 2002; Boucher *et al*., 2018). NLRP3 inflammasome assembly and activity is controlled by two stages, priming and activation.

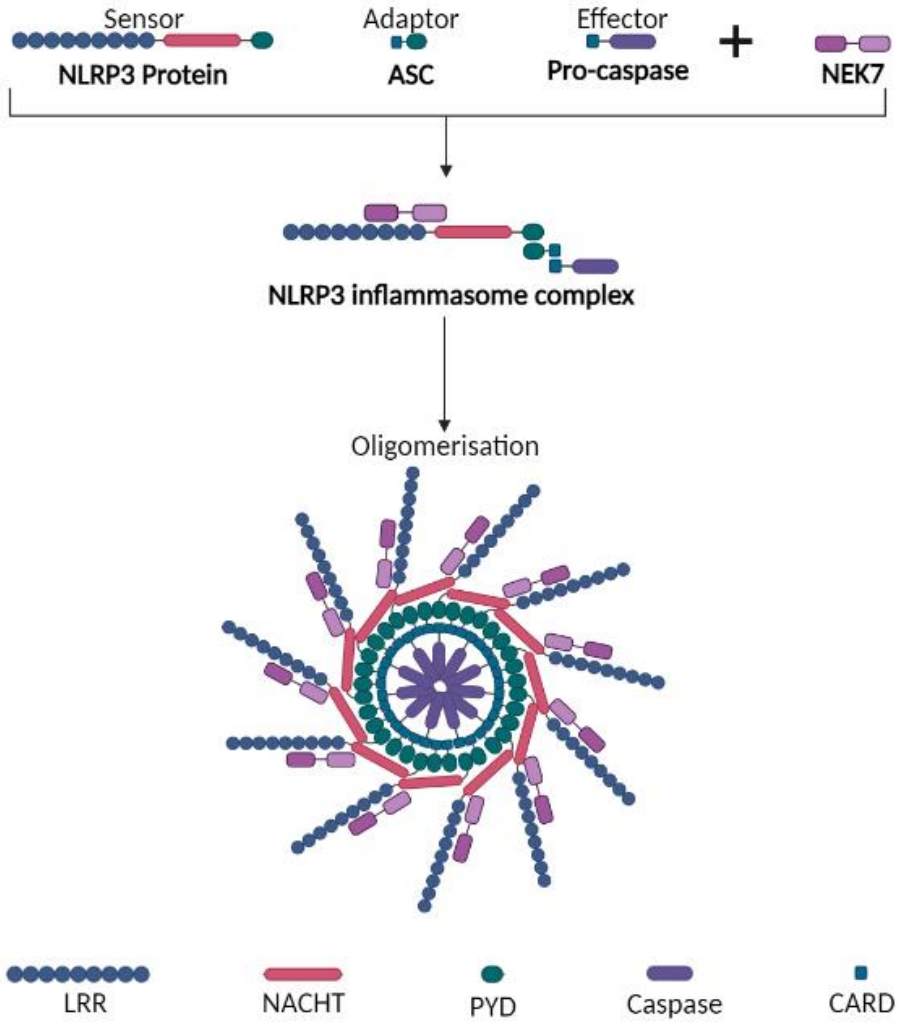


Fig 3.2. NLRP3 inflammasome structure and assembly. The NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome consists of a sensor NLRP3 protein, adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and an effector pro-caspase. The NLRP3 protein contains three parts, a leucine rich repeat (LRR), ATPase containing NACHT (NLRP3 nucleotide binding and oligomerisation) domain and a pyrin (PYD) domain. When the NLRP3 inflammasome is activated, ASC binds to the NLRP3 protein via PYD-PYD polymerisation and subsequently recruits pro-caspase via the caspase activation and recruitment domain (CARD). The NLRP3 inflammasome complex then oligomerises and assembles into a circular cage structure. Oligomerisation is mediated by NEK7 (never in mitosis gene A (NIMA)-related kinase 7), which binds to the LRR and NACHT domain of the NLRP3 protein. Figure created using BioRender.com.

3.1.4. NLRP3 priming

Inflammasomal priming is needed to increase gene and protein expression of inflammasomal components, with post-translational modifications that maintain NLRP3 in an inactive configuration (Fig 3.3) (Yang *et al*., 2017; Swanson *et al*., 2019; Duez & Pourcet, 2021). In the context of bacteria, PRRs on macrophages bind to PAMPs on bacteria to initiate the inflammasome pathway. There are six classes of PRRs, of which, TLRs are responsible for bacterial recognition (Gong *et al*., 2020; Duez & Pourcet, 2021). TLRs are type I transmembrane proteins comprised of an extracellular LRR responsible for PAMP recognition, a transmembrane helix, and an intracellular TIR required for the activation of downstream signal transduction pathways (El-Zayat *et al*., 2019).

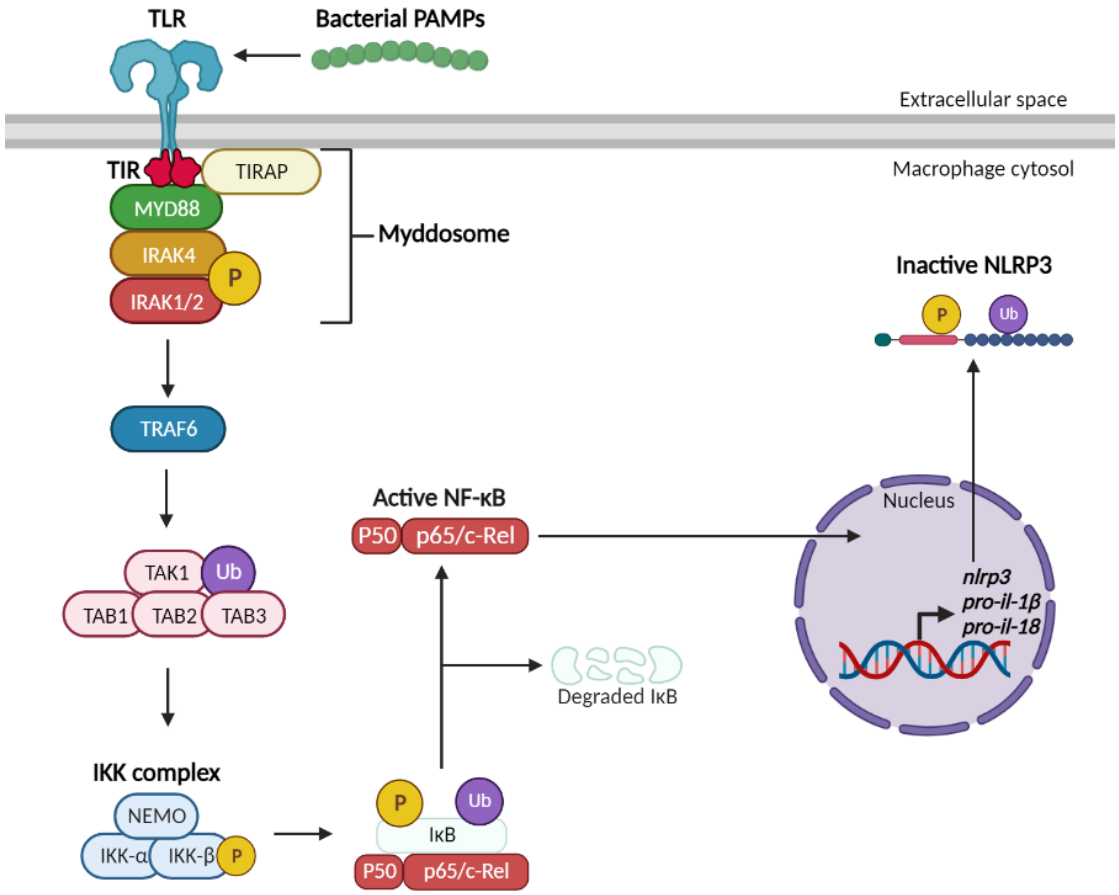


Fig 3.3. Priming of the NLRP3 inflammasome. Bacterial PAMPs (pathogen-associated-molecular patterns) interact with TLRs (toll-like receptors) expressed on the extracellular membrane of macrophages. Upon recognition, TLRs dimerise causing the intracellular TLR TIR (toll-interleukin 1 receptor) domain to dimerise. TIRAP (TIR domain-containing adaptor protein) associates with the plasma membrane and the TLR-TIR domain, forming a bridge to allow the MyD88 (myeloid differentiation 88) adaptor protein to associate and activate. MyD88 recruits IRAK4 (interleukin-1 receptor associated kinase 4) which autophosphorylates (P), subsequently activating IRAK1/2 and creating a large signalling complex called the Myddosome. IRAK1 activates TRAF6 (TNF receptor-associated factor 6), which ubiquitinates (Ub) TAK1 (transforming growth factor-β-activated kinase 1) to form a complex with TAB1, 2 and 3 (TAK1 binding protein), activating TAK1. This complex activates the IKK complex consisting of three subunits: NEMO, IKKα and IKKβ. The activated IKK (inhibitor of nuclear factor-κB (IκB) kinase) complex phosphorylates and ubiquitinates IκB causing it to dissociate from NF-κB (nuclear factor kappa B). Active NF-κB translocates into the nucleus and causes increased transcription of *nlrp3* and inactive pro forms of the pro-inflammatory cytokines *il-1β* and *il-18*. NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) undergoes post translational modifications in the form of phosphorylation and ubiquitination in order to maintain its inactive configuration, ready for an activation signal to promote NLRP3 inflammasome assembly and activation. Figure created using BioRender.com.

TLR4 binds the Gram-negative bacterial cell wall component LPS and TLR2 binds to LTA and lipopeptides in Gram-positive bacteria. Both these TLRs are expressed on the BMMO extracellular membrane (Gong *et al*., 2020; Duez & Pourcet, 2021). TLR2 forms dimeric complexes with either TLR1 or TLR6, which recognise triacyl lipopeptides or diacyl lipopeptides and LTA respectively (Fig 3.4) (Botos *et al*., 2011). TIR dimerisation of TLRs is recognised by TIR domains on one of the five adaptor proteins MyD88 (myeloid differentiation 88), TIRAP (TIR domain-containing adaptor protein) also known as MAL (MyD88-adaptor like), TRIF (TIR domain-containing adaptor protein inducing IFN-β), TRAM (TRIF-related adaptor molecule) or SARM (sterile α- and armadillo-motif-containing protein), which then trigger downstream signalling pathways. TLR2 and TLR4 indirectly recruit MyD88 through TIRAP association (Lin *et al*., 2010; Patterson & Werling, 2013; El-Zayat *et al*., 2019; Rajpoot *et al*., 2021). TIRAP consists of two domains, a phosphatidylinositol 4,5-bisphosphate (PIP_2) binding domain (PBD) allowing TIRAP to interact with the plasma membrane and a TIR domain. Once associated with the plasma membrane, tyrosine kinases, such as Bruton's tyrosine kinase (BTK), facilitate tyrosine phosphorylation in both the TIR domains of TLRs and TIRAP. Following TIRAP-TIR binding to TLR-TIR, the TIR domain of MyD88 then associates with TIRAP-TIR. Ultimately TIRAP forms a "bridge" between the TIR domains of TLRs and MyD88 (Bernard & O'Neill, 2013; Balka & Nardo, 2019; Rajpoot *et al*., 2021).

MyD88 has a tripartite structure containing a death domain, an intermediate domain and a TIR domain. MyD88 recruits IRAK4 (interleukin-1 receptor associated kinase 4) which becomes activated through autophosphorylation, subsequently activating both IRAK1 and IRAK2 creating a large signalling complex called the Myddosome. Once IRAK1 is activated it induces association and activation of TRAF6 (TNF receptor-associated factor 6), which simultaneously triggers three transduction pathways, one of which is involved in inflammasomal priming. TRAF6 ubiquitinates the MAPKKK protein TAK1 (transforming growth factor-β-activated kinase 1), resulting in TAK1 forming a complex with TAB1 (TAK1 binding protein 1), 2 and 3 which interact with TRAF6 to activate TAK1 (Jiang *et al*., 2002; Kollewe *et al*., 2004; Chen, 2012; Ajibade *et* al., 2013; Balka & Nardo, 2019). TAK1 binds to the IKK complex (inhibitor of nuclear factor-κB (IκB) kinase), consisting of two catalytic subunits, α and β, and a regulatory subunit γ (NEMO). TAK1 phosphorylates and activates IKKβ. The IKK complex can then phosphorylate IκB, an inhibitory protein bound to NF-κB keeping this transcription factor in an inactive state. Once, phosphorylated, IκB dissociates from NF-κB and is degraded through ubiquitination (Akira *et al*., 2006; Kawai & Akira, 2010). This activates NF-κB which translocates into the

nucleus and increases transcription of *nlrp3*, *pro-il-1β* and *pro-il-18* (Bauernfeind *et* al., 2009; Franchi *et al*., 2009).

Post-translational modifications, such as phosphorylation and ubiquitination, are then needed to maintain NLRP3 in an inactive configuration so the inflammasomal components are at activating threshold (Yang *et al*., 2017; Duez & Pourcet, 2021). For example, JNK1 (c-Jun N-terminal protein kinase 1) phosphorylates NLRP3 at Ser198 to induce self-association of NLRP3 to promote inflammasome assembly (Seok *et al*., 2021). Therefore, when there is an activation signal, NLRP3 can act rapidly in promoting inflammasome assembly and subsequent activity (Swanson *et al*., 2019).

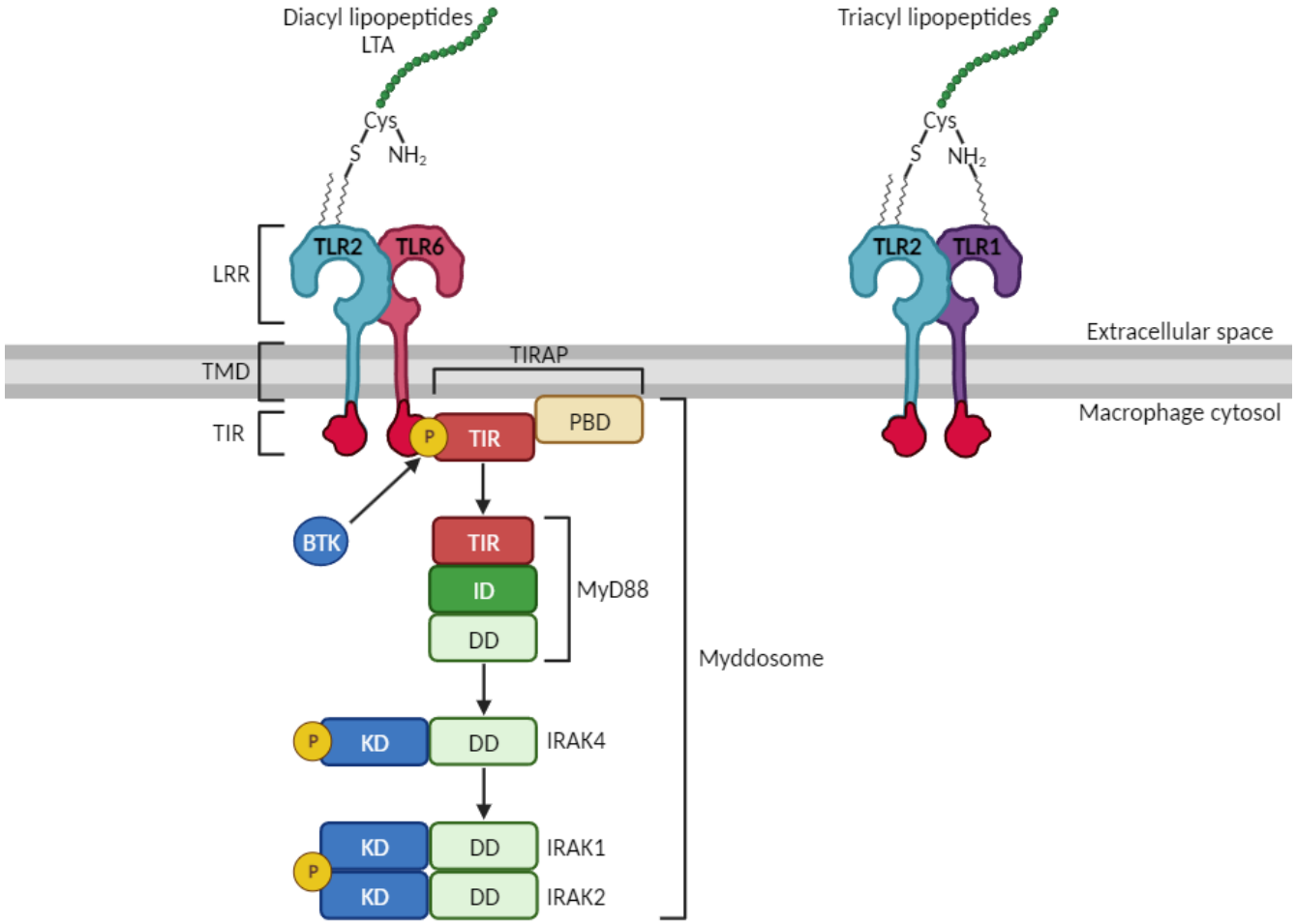


Fig 3.4. TLR2 structure, binding and myddosome. TLR2 (toll-like receptor 2) consists of an extracellular LRR (leucine rich repeat), TMD (transmembrane domain) and an intracellular TIR (toll-interleukin receptor). TLR2 forms dimeric complexes with TLR6 or TLR1, which recognise diacyl lipopeptides and lipoteichoic acid (LTA) or triacyl lipopeptides respectively. Upon binding to TLR2, the TLR2-TIR domain dimerises and binds to the TIR domain of TIRAP (TIR domain-containing adaptor protein) which is facilitated through phosphorylation (P) of both TIR domains by BTK (Bruton's tyrosine kinase). TIRAP is in close proximity to TLR2-TIR due to expression of a phosphatidylinositol 4,5-bisphosphate (PIP_2) binding domain (PBD) allowing TIRAP to interact with the plasma membrane. The TIR domain of MyD88 (myeloid differentiation 88) then binds to TIRAP-TIR. MyD88 is a tripartite protein that also consists of an intermediate domain (ID) and a death domain (DD) which recruits IRAK4 (interleukin-1 receptor associated kinase 4) via its death domain. IRAK 4 becomes autophosphorylated through its kinase domain (KD), subsequently activating both IRAK1 and IRAK2, creating the large signalling complex called the Myddosome. Figure created using BioRender.com.

3.1.5. NLRP3 activation signals

There are a vast variety of different stimuli that can act as NLRP3 inflammasome activation signals (Fig 3.5). These signals can occur from extracellular stimuli or due to the accumulation of molecules intracellularly. Phagocytosed compounds can be trafficked to the lysosome where they result in damage causing the release of cathepsins (Hornung *et al*., 2008). Ion fluxes have also been reported as regulators of inflammasome activation. Notably, calcium influx promotes NLRP3 inflammasome assembly (Murakami *et al*., 2012; Muñoz-Planillo *et al*., 2013); potassium efflux causes NEK7 dependent NLRP3 oligomerisation (Muñoz-Planillo *et al*., 2013) and chloride efflux induces ASC polymerisation (Green *et al*., 2018). Inflammasome priming can lead to extracellular ATP damaging the mitochondria releasing mtDNA into the cytosol that, in turn, becomes oxidised and can interact with NLRP3, activating the inflammasome complex (Nakahira *et al*., 2011; Shimada *et al*., 2012).

Once the inflammasome receives both priming and activating signals, pro-IL-1β and pro-IL-18 are cleaved into their active forms by caspase-1 and are secreted out of the BMMO to initiate an immune response (Yang *et al*., 2017; Swanson *et al*., 2019; Duez & Pourcet, 2021). IL-1β has many functions, one of which is to activate neighbouring macrophages to enhance the amount of IL-1β secreted. IL-1β also binds to epithelial cells which become activated and lead to the recruitment of other immune cells, such as neutrophils and lymphocytes to the site of infection. IL-18 interacts with T-cells to induce IFN-γ secretion that binds to receptors on macrophages, further increasing macrophage stimulation (Dinarello, 2009; Kelley *et al*., 2019).

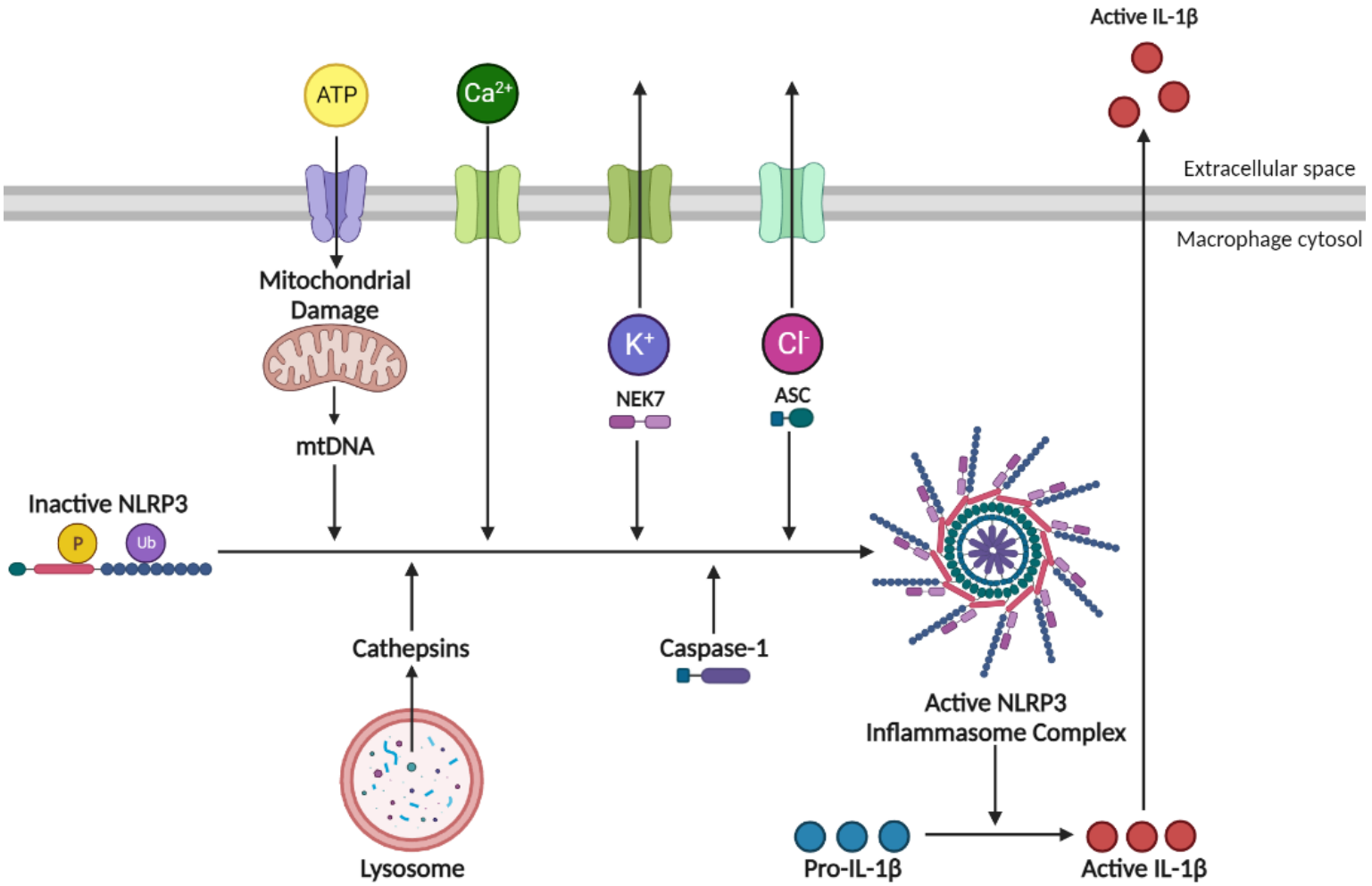


Fig 3.5. Activation of the NLRP3 inflammasome. Once NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) is primed, the inflammasome can become assembled and activated. This can occur through a variety of different stimuli. Phagocytosed compounds can be trafficked to the lysosome where they cause the release of cathepsins. Extracellular ATP can enter the cytosol and cause mitochondrial damage resulting in the release of mtDNA that interacts with NLRP3. Calcium ion influx promotes NLRP3 inflammasome assembly. Potassium ion efflux causes NLRP3 oligomerisation in a NEK7 (never in mitosis gene A (NIMA)-related kinase 7) dependent manner. Chloride ion efflux induces ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) polymerisation. Activated NLRP3 inflammasome complex allows caspase-1 to cleave pro-IL-1β into its active form which is secreted out of the macrophage to initiate an immune response. Figure created using BioRender.com.

3.1.6. S. pyogenes, S. pneumoniae and S. aureus stimulate the NLRP3 inflammasome

S. pyogenes has been shown to prime the NLRP3 inflammasome either through binding to TLR2 on the extracellular surface of macrophages or following internalisation into endosomes through TLR8/9. This results in downstream signalling pathways that activate NF-κB and increase transcription of *nlrp3*, *pro-il-1β* and *pro-il-18*. NLRP3 inflammasome activation signals can be provided by several *S. pyogenes* factors, most notably, the cytolysins Streptolysin O and S, which permeabilise the macrophage plasma membrane resulting in potassium efflux (Richter *et al*., 2021). Caspase-1 activation in macrophages following *S. pyogenes* infection activates NF-κB independent of TLR signalling by Streptolysin O (Harder *et al*., 2009). The ADP-ribosyl transferase, SpyA, and the release of the M1 bacterial surface protein have also been identified as NLRP3 activation signals, however, the mechanism of action of these compounds remains unknown (Richter *et al*., 2021). The extracellular cysteine protease streptococcal pyrogenic exotoxin B (SpeB) has been found to bypass inflammasome activation and directly cleave pro-IL-1β to produce biologically active IL-1β (Kapur *et al*., 1993).

Similarly, *S. pneumoniae* has been shown to cause increased *pro-IL-1β* transcription by priming NLRP3 through the TLR2-MyD88 pathway in macrophages (Rodriguez *et al*., 2019). Additionally, the NLRP3 inflammasome and caspase-1 were found to be essential for *S. aureus* mediated release of IL-1β and IL-18 from MAC-T cells (Wang *et al*., 2022).

Previous studies have suggested that *S. uberis* activates the NLRP3 inflammasome, however, there has been limited research into the mechanism by which *S. uberis* primes and activates the BMMO NLRP3 inflammasome. Results to date indicate that SUB1154 is involved in this inflammasomal pathway, therefore, it is important to determine the precise function of the SUB1154 protein in this pathway.

3.2. Methods

3.2.1. Recombinant SUB1154 protein purification

Recombinant SUB1154 protein (rSUB1154; Glu61-Thr1113-Arg-Ser 6[His]-TAA) was constructed to accommodate predicted propeptide processing/autocatalytic cleavage at the N-terminus as described for C5a peptidase from *Streptococcus pyogenes* (Anderson *et al*., 2002). The C-terminus of the recombinant protein matched the predicted sortase-cleaved and was coupled to a 6[His] affinity tag. The proteolytically compromised (rSUB1154NP) construct with mutation Ser496Ala was engineered from rSUB1154 by reverse PCR with the aim of disrupting the Ser, His, Asp catalytic triad common to most serine proteases. The sense and antisense Ser mutation oligonucleotide primers used were 5'- CAAAGATGTCAGGAACTGCTGCT GCAAGTCC and 5'-GCATGTGGACTTGCAGCAGCAGTTCCTGACATC respectively. Both recombinants were constructed by Dr Phillip Ward and supplied by Professor James Leigh.

The rSUB1154 and rSUB1154NP proteins were purified from *E. coli* hosts using the pQE-1 expression plasmid containing an ampicillin resistance marker gene, a T5 promoter and a LacO operon. *E. coli* host strains were inoculated in Luria-Bertani (LB) broth (Sigma, 51208) with 50 µg/mL of ampicillin (Sigma, A9518) and incubated overnight at 37°C. The inoculate was then diluted 1:50 with 100 mL prewarmed LB broth containing ampicillin in a 500 mL conical flask and grown in a shaking incubator (GFL Incshaker, T3031) for 3h at 160 RPM and 37°C. 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, I6758) was added and incubation was continued for a further 2h. Cells were then harvested by centrifugation at 4,500xg for 15 min at 4°C. The cells were washed in sterile PBS and the pellets were frozen at -20°C overnight.

The harvested bacterial cells were thawed on ice and resuspended in 20 mL of CelLytic™ B Cell Reagent (Sigma, B7435) per gram of cell pellet. Proteolytic degradation was prevented by the addition of 1 mM cOmplete™ protease inhibitors (Roche, 11697498001). To completely lyse the bacterial cells the suspension was incubated shaking at 160 RPM at room temperature for 15 min and any debris was removed by centrifugation at 16,000xg for 10 min. The supernatants were collected and centrifuged again at 16,000xg for 10 min. The resulting supernatants were filter sterilised using a 0.45 µM filter (Millex®HA, SLHAM33SS) and purification of the recombinant proteins achieved using HisPur™ Ni-NTA Chromatography protein purification cartridges (Thermo Scientific, 90098) following the manufacturer's instructions. Briefly, at a flow rate of 1 mL/min, the columns were flushed with 10 mL of deionised water to remove the storage solution and equilibrated with 10 mL of wash buffer (10 mM imidazole (Sigma, I5513-5G) in PBS). After loading the filtered supernatants, unbound proteins were removed using 10

mL of wash buffer and the target proteins eluted using elution buffer (250 mM imidazole in PBS). The eluants were collected in 1 mL fractions and those containing the SUB1154 proteins were determined by SDS-PAGE (3.2.2 below).

Removal of imidazole was achieved by dialysis using Slide-A-Lyzer dialysis cassettes (Thermo Scientific, 2160728) following the manufacturer's instructions. Cassette membranes were hydrated in 1L of ultrapure water for 2 min. Fractions containing the soluble rSUB1154 proteins were added to the cassettes and suspended, gently stirring, in the water for ~20h at 4°C with two changes of water at 2h and 4h. Purified rSUB1154 proteins were extracted from the dialysis cassette and any endotoxin was removed using Pierce® High-Capacity Endotoxin Removal Spin Columns (0.50 mL Thermo Scientific, 88274). Columns were washed with ultrapure water and then equilibrated with endotoxin free PBS. Purified rSUB1154 proteins were added to the columns at a flow rate of 10-15 mL/h. An additional 1 mL of PBS was put through the pump and 1 mL directly to the resin to ensure elution of proteins. Concentration of the rSUB1154 proteins were determined using a spectrophotometer/fluorometer (Denovix, DS-11 FX+) and stored with 10% glycerol (Fisher Chemical, G/0650/08) at -80°C.

3.2.2. SDS-PAGE

Samples were mixed 1:1 with (2X) SDS sample buffer (Novex, 2201443) and heated to 95°C for 5 min. Samples and blue prestained protein standard, broad range ladder (11-250 kDa; New England Biolabs, P7718S) were loaded onto 10% Mini-PROTEAN TGX Stain-free gels (Biorad, 4568036) with running buffer containing 25 mM Tris, 200 mM glycine (Thermo Fischer, 28363) and 0.1% SDS and electrophoresis conducted at 120 V. Gels were exposed to UV for 5 min and image captured using a Biorad ChemiDoc™ Imaging System (Fig SI2).

3.2.3. Bacterial culturing conditions

S. uberis strains 0140J and SUB1154 deletion mutant (0140JΔ*sub1154*) were cultured following the protocol outlined in 2.2.3. *S. uberis* 0140JΔ*sub1154* was generated as previously described by allelic exchange mutagenesis (Leigh *et al*., 2010).

3.2.4. S. uberis strain 0140JΔsub1154 confirmation

S. uberis strain 0140JΔ*sub1154* DNA was extracted and underwent PCR following the same protocols outlined in 2.2.4 and 2.2.5. Primers (Sigma) used are described below in Table 3.1 (10 µM in DNase free water). Thermocycling protocol described in Table 2.2. Gel electrophoresis was conducted on the PCR products as described previously (2.2.6).

Table 3.1. DNA PCR primers.

Gene target*	Sequence	Function	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)
S. uberis sub1154 (internal)	AM946015.1	Serine protease	ACAAAGTTGAAAA GGGGCGT	CGCCATTAGGTGA AAGTGCT	60	573

*Gene according to the genomic sequence of *S. uberis* strain 0140J (Ward *et al*., 2009).

3.2.5 BMMO challenge

Following isolation, BMMOs were challenged with various stimuli as described in the associated narrative text (section 3.3) and IL-1β measured after 20h. The stimuli used were: heat-killed *S. uberis* strains 0140J and 0140JΔ*sub1154* at an MOI of 50:1 bacterium:BMMO; LPS (10 ng/mL; Millipore, LPS25); rSUB1154 and rSUB1154NP (section 3.2.1); inflammasome primer, Pam3CSK4 (Torcis, 4633); inflammasome activator, silica (Sigma, 421553); cell entry inhibitor, Cytochalasin D (CyD) (10 µM; Torcis, 1233); the TLR2 inhibitors C29 (100 µM; Adooq Bioscience, A17160) and MMG 11 (100 µM; Tocris, 6858), the NLRP3 inhibitor MCC950 (1 µM; Apexbio, B7946); the caspase-1 and caspase-4 inhibitor AC-YVAD-CMK (50 µM; Sigma, SML0429) and the caspase-1 and caspase-3 inhibitor Z-YVAD-FMK (100 µM; Seltechchem, S7391).

3.2.6. ELISA

Detection of bovine IL-1β ELISA was achieved using the Invitrogen Reagent Kit (ESS0027) following the manufacturer's instructions, as previously described (section 2.2.8).

3.2.7. Caspase-1 activity assay

Caspase-1 activity was determined using the Abcam colorimetric kit (ab273268). Following BMMO challenge with stimuli for 20 and the removal of medium, BMMOs were lysed in 50 µL of the provided buffer and incubated on ice for 10 min. Samples were centrifuged for 1 min at 10,000xg and the supernatant protein concentration was adjusted to 150 µg using the dilution buffer. Reaction buffer and substrate were added and incubated at 37°C for 1.5h. Substrate conversion was determined by measurement of absorbance at 400 nm and caspase-1 activity units interpolated from a standard curve.

3.2.8. RNA extraction and Real-Time quantitative Reverse Transcription PCR (RT qRT-PCR)

RNA was extracted from BMMOs using the Qiagen RNeasy Mini Kit (74104). RNeasy lysis buffer and 70% ethanol in equal volumes were added to the BMMOs and centrifuged in spin columns at 8,000xg for 15 sec. The membrane-bound RNA was washed in RW1 buffer, and the washing buffer removed by centrifugation (8,000xg for 15 sec). RNA was then washed with RPE buffer twice and the washing buffer removed by centrifugation (8,000xg for 15 sec and subsequently 2 min) prior to elution of the RNA in RNase free water. RNA was precipitated by the addition of 0.1 volume of sodium acetate (Sigma, W302406), 3 volumes of ethanol and 2 µL of glycoblue coprecipitate (Invitrogen, AM9515) and incubation overnight at -20°C. Precipitated RNA was collected by centrifugation (15,000xg; 15 min), washed in 70% ethanol and pelleted by centrifugation at 15,000xg for 3 min. The RNA pellets were subsequently dried, resuspended in RNase free water and the concentration determined and adjusted to approximately 15 ng/µL using a spectrophotometer/fluorometer (Denovix, DS-11 FX+).

RT qRT-PCR of the RNA was completed using Luna® Universal One-Step RT-qPCR Kit with the primers (Sigma) listed in Table 3.2 (10 µM in DNase free water). 20 µL reactions were performed in microamp optical 96-well reaction plates (Applied Biosystems, N8010560) on a Biorad CFX connect real-time system instrument (788BR7113). Biorad CFX maestro software was used to complete the SYBR® scan mode protocol using the thermocycling protocol below (Table 3.3).

Table 3.2. RT qRT-PCR RNA primers.

Gene target	Sequence	Function	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)	Gene size (bp)
actb	NM_173979.3	Part of the actin cytoskeleton; involved in cell shape and movement.	GCAGGAGTACGATGAGTCCG	TGTCACCTTCACCGTTCCAG	64.8 66.3	220	1948
gapdh	NM_001034034.2	Enzyme involved in the breakdown of glucose.	GCCCTCTCAAGGGCATTCTA	ATTCTCAGTGTGGCGGAGAT	65.4 63.7	297	1279
rpl13a	NM_001015543.2	Component of the 60S ribosomal subunit.	GGCTCGCAAGATCCGTAGAC	ACAGGATAAGCTTGGAGCGG	66.0 65.4	293	926
casp1	XM_002692921.5*	Proteolytically cleaves precursor forms of IL-1β and IL-18.	GGAAGCCATGGCCGACAA	GCCAGGTGGGAGTCTTCTTC	69.6 65.1	258	2002
nek7	XM_003587112.5†	Involved in NLRP3 activation.	TTTGGTACCAGTTCCCCAGG	TACGCATACAAGCACGGTGT	65.8 64.1	206	4023
nf-κb	NM_001192970.1‡	Transcriptional regulator of the immune response.	ATACCTGCCAGATGAAAAGGACAC	TCGGTAGCATGGCTGAAGCAG	66.1 69.6	296	1830
nlrp3	NM_001102219.1	Main component of the inflammasome complex.	CTTTCTGGACTCTGACCGGG	ATGGCCCATGCCTTCTCTTC	66.0 67.5	236	3715
pro-il-1β	NM_174093.1	Pro-inflammatory cytokine.	CGACTGCCTTCCCTGCATTA	TTTTTCACAGATGCGCCTGC	67.3 68.4	294	1736
pro-il-18	XM_005215801†	Pro-inflammatory cytokine.	TGCTCTCCAATGCTTTCAGCG	GTGATCTGATTCCAGGTCATCATTT	69.7 66.0	250	801
tlr2	NM_174197.2	Pathogen recognition.	TGTAAACTTGAGAGTGGAGGTCA	TTTCACACCTGCCGTGAGAC	62.6 66.4	200	3513
tlr2	NM_174197.2	Pathogen recognition.	CTTGAGAGTGGAGGTCAAATCACT	GTGTGAATTTCATTGGCCCCC	64.6 69.3	298	3513
nf-kb	NM_001192970.1‡	Transcriptional regulator of the immune response.	GGCAAGAACGCAGACCTTTG	GGGTTGGAGACAACAGGAGG	67.0 65.8	241	1830
nlrp3	NM_001102219.1	Main component of the inflammasome complex.	CTTTCTGGACTCTGACCGGG	ATGCCTTCTCTTCCCCGTTG	66.0 67.4	229	3715
casp1	XM_002692921.5*	Proteolytically cleaves precursor forms of IL-1β and IL-18.	AGGCTAAGAAGGGGAAGCCA	GCCAGGTGGGAGTCTTCTTC	66.0 65.1	270	2002

*Predicted transcript variant X2. †Predicted transcript variant X1. ‡ REL proto-oncogene, NF-κB subunit. ACTB, actin beta; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL13a, ribosomal protein 13; CASP1, pro-caspase-1; NEK7, never in mitosis gene A (NIMA)-related kinase 7; NF-κB, nuclear factor kappa B; NLRP3, nod-like receptor (NLR) family pyrin domain containing 3; Pro-IL-1β, interleukin-1 beta precursor; Pro-IL-18, interleukin-18 precursor; TLR2, toll-like receptor 2.

All primers were supplied by Sigma and were designed using the mRNA sequence on the NCBI database (specific sequence code referenced in table). The first three rows are the reference genes, and the following 7 rows are the target genes. The last 4 rows are details of initial primers for target genes that were shown to be unsuitable and redesigned (due to same values as blank). Redesigned primers were edited to span the exon-exon junction to avoid amplification of any residual DNA contamination. The melting temperature (Tm) for the forward primer is the first value and the reverse primer is the second value. Product and gene sizes are given in base pairs (bp).

Table 3.3. RT qRT-PCR thermocycling protocol.

Cycle step	Temperature	Time	Cycles
Reverse transcription	55°C	10 min	1
Initial denaturation	95°C	1 min	1
Denaturation Extension	95°C 62°C	10 sec 30 sec + plate read	40
Melt curve	65°C to 95°C in 0.5°C increments	Increment every 5 sec + plate read	1

3.2.9. Statistical analysis

Data was analysed using GraphPad Prism 10.0.3 (GraphPad Software, 2023). Normality was assumed due to the low N value and data was statistically analysed using a one-way ANOVA followed by Tukey multiple comparisons post hoc test. A value of $P \leq 0.05$ was considered to indicate a statistically significant difference.

The RT qRT-PCR data was analysed by first determining the mean Cq value for each condition using the three reference genes (ACTB, GAPDH, RPL13a). Then, for each condition, the mean Cq was deducted from the corresponding test gene Cq to determine the ΔCq. The ΔCq was then averaged for the no treatment condition (one value for each test gene). After that, the ΔΔCq was determined by deducting the mean no treatment ΔCq from each of the conditions. From these values, the 2^-ΔΔCq (Relative Quantification, RQ) was calculated. Finally, the log-2 was calculated for all the RQ values. Values at 0h were used to determine baseline transcription (0 Log_2).

3.3. Results

3.3.1. BMMOs challenged with S. uberis produce IL-1β via the NLRP3 inflammasome

As the macrophages used in this study were isolated using a method different to that used previously (Archer *et al*., 2020), in which cells were isolated by immobilisation through CD14, it was necessary to confirm that functionally the cells to be used in this study showed responses consistent with the cell population used by Archer *et al*., 2020. There was ablation of IL-1β secretion when BMMOs were challenged with either LPS or *S. uberis* strain 0140J in the presence of the NLRP3 (MCC950), caspase-1 and caspase-4 (AC-YVAD-CMK) and caspase-1 and caspase-3 (Z-YVAD-FMK) inhibitors (Fig 3.6). Using trypan blue, the cell viability of the isolated BMMOs challenged with each of the inflammasome inhibitors was measured at >90%. Therefore, IL-1β production from BMMOs following stimulation with *S. uberis* strain 0140J occurs via the NLRP3 inflammasome.

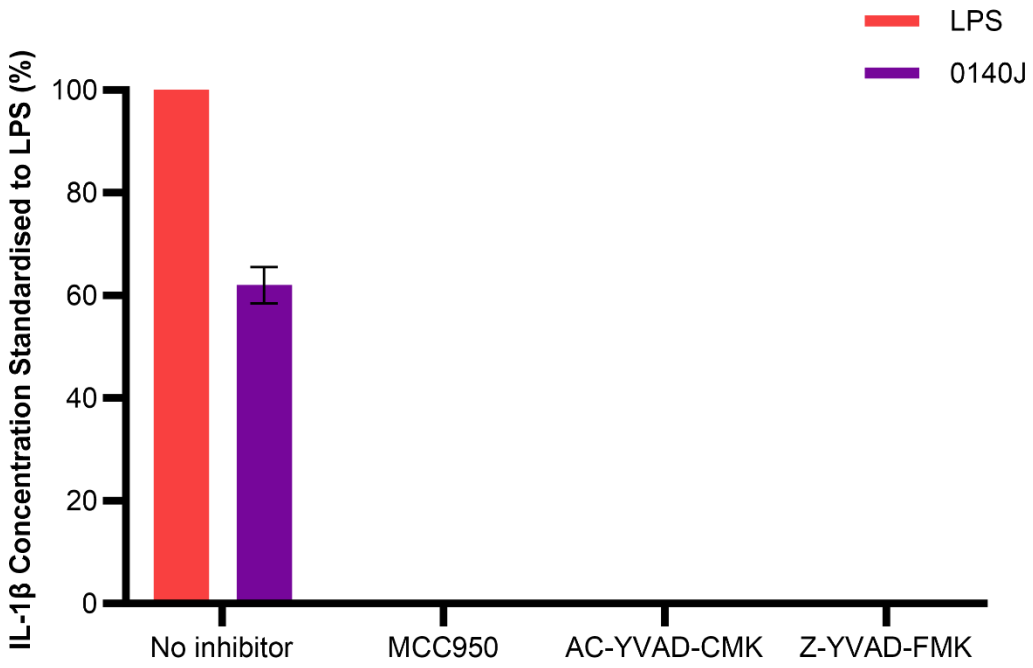


Fig 3.6. Ablation of IL-1β production from BMMOs following NLRP3 inflammasome inhibition. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were incubated in the presence of the inflammasome inhibitors: MCC950, 1 µM (NLRP3 inhibitor); AC-YVAD-CMK, 50 µM (caspase-1 and caspase-4 inhibitor) or Z-YVAD-FMK, 100 µM (caspase-1 and caspase-3 inhibitor) for 1h before challenge with either heat-killed *S. uberis* strain 0140J at a multiplicity of infection (MOI) of 50:1 *S. uberis* strain 0140J:BMMO or LPS (10 ng/mL). Supernatants were collected at 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control. Data is presented as N=3±SD.

3.3.2. Challenge of BMMOs with different concentrations of the recombinant SUB1154 proteins

Previous research has shown that the SUB1154 protein is involved in the inflammatory response to *S. uberis* in BMMOs (Archer *et al*., 2020). Recombinant SUB1154 with the predicted protease domain intact (rSUB1154) or removed (rSUB1154NP) were purified and BMMOs were challenged at varying concentrations, 2-fold dilution from 20 µM to 2.5 µM (Fig 3.7A) and subsequently in a range of concentrations from 20 µM to 0.002µM (Fig 3.7B). BMMOs challenged with both rSUB1154 and rSUB1154NP at 20, 10 and 5 µM of either protein produced low levels of IL-1β with no differences detected in the response between the two proteins. Stimulation of BMMOs with all other protein concentrations found no detectable IL-1β. Subsequent experiments challenged BMMOs with 0.002 µM of SUB1154 proteins to correspond more physiologically realistic concentrations.

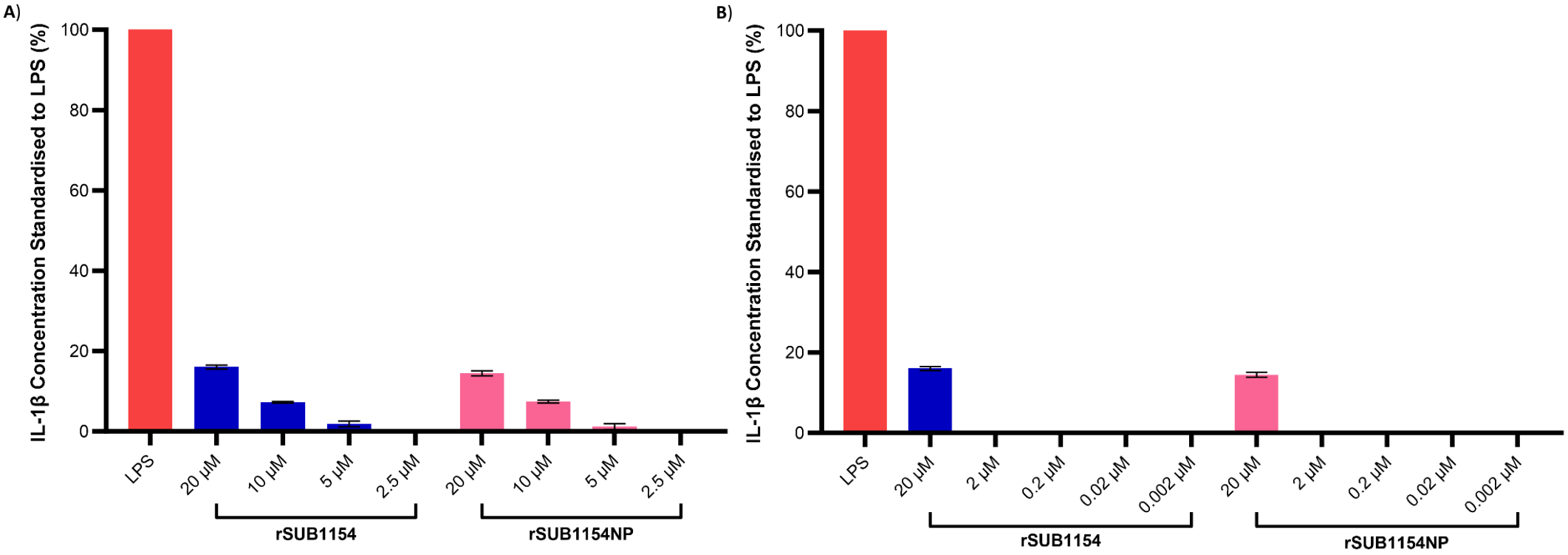


Fig 3.7. IL-1β production from BMMOs stimulated with the *S. uberis* SUB1154 protein. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with purified recombinant rSUB1154 or rSUB1154NP (proteolytically compromised) protein at a 2-fold dilution from 20 µM to 2.5 µM (**A**) and a 10-fold dilution from 20 µM to 0.002 µM (**B**). Supernatants were collected 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD.

3.3.3. Confirmation of S. uberis 0140J SUB1154 deletion mutant

SUB1154 was deleted in an *S. uberis* strain 0140J isolate (0140JΔ*sub1154*); extracted DNA from *S. uberis* strain 0140JΔ*sub1154* underwent PCR and gel electrophoresis to ensure validity, alongside *S. uberis* strain 0140J. The PCR primers amplified *sub0888* (unique to *S. uberis*) were found in both *S. uberis* strain 0140J and 0140JΔ*sub1154* (Fig 3.8A), whereas PCR primers amplified the *sub1154* product from *S. uberis* strain 0140J but not from 0140JΔ*sub1154*, confirming SUB1154 deletion (Fig 3.8B).

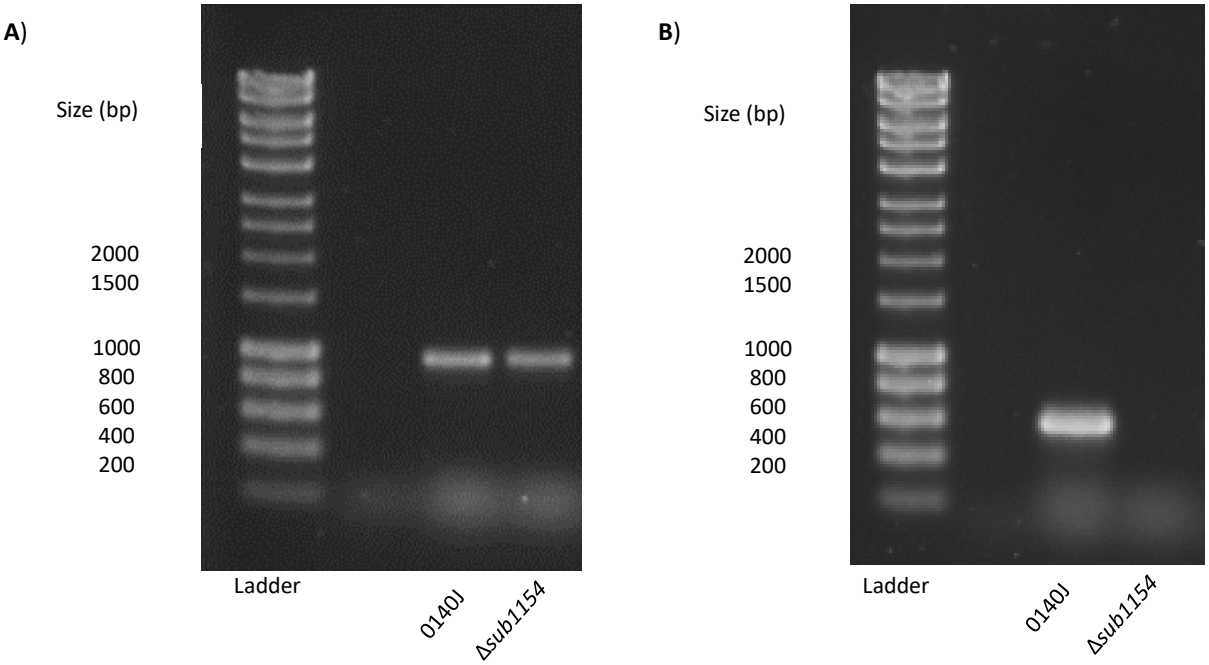


Fig 3.8. Confirmation of the *S. uberis* SUB1154 mutant. DNA was extracted from *S. uberis* strains 0140J and 0140JΔ*sub1154* cultures, amplified by PCR using primers for SUB0888, encoding a sortase anchored protein, (**A**) or SUB1154 (**B**) and imaged through gel electrophoresis. SUB0888 is considered unique to *S. uberis* and produces a product size of 974 base pairs (bp). SUB1154 produces a product size of 573 bp.

3.3.4. S. uberis SUB1154 protein is involved in the production of IL-1β from BMMOs

To determine the effect of SUB1154 in the presence and absence of the bacterial cells, BMMOs were challenged with combinations of *S. uberis* 0140JΔ*sub1154* and purified rSUB1154/NP (Fig 3.9). In the absence of stimulation (NT) there was no IL-1β detected. *S. uberis* strain 0140J elicited 60% IL-1β compared to that obtained from LPS stimulation. When SUB1154 was absent and BMMOs were challenged with the deletion mutant (0140JΔ*sub1154*) alone, there was no IL-1β produced. BMMOs challenged with both the rSUB1154 and *S. uberis* 0140JΔ*sub1154* resulted in partial restoration of IL-1β production, however, this was significantly less when compared to treatment with *S. uberis* strain 0140J (P<0.0001). Despite there being some

degree of IL-1β production, challenge with the combination of rSUB1154NP and *S uberis* 0140JΔ*sub1154* resulted in significantly less IL-1β production compared to rSUB1154 and *S. uberis* 0140JΔ*sub1154* (*P*<0.0001) (Fig 3.9).

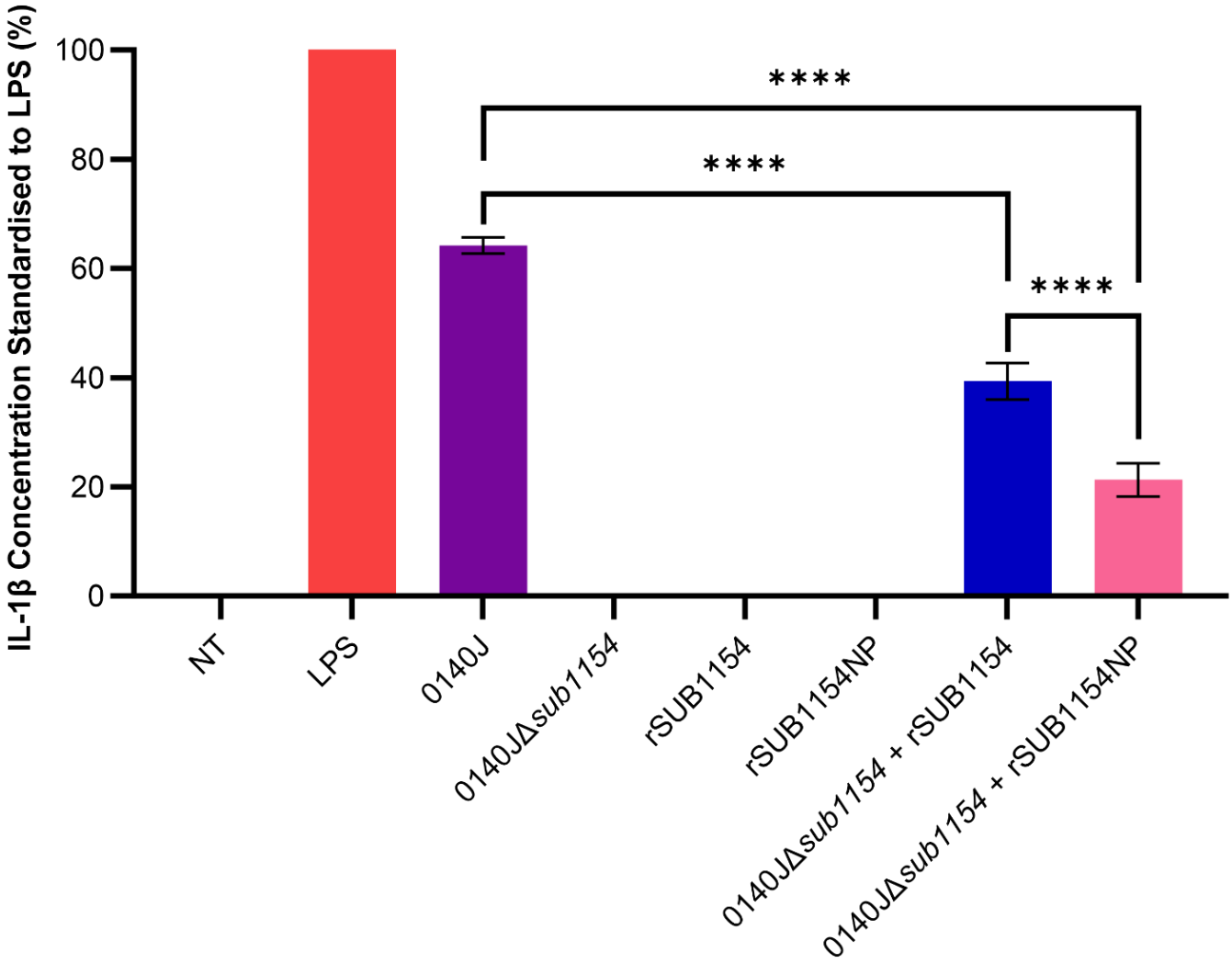


Fig 3.9. *S. uberis* SUB1154 protein is involved in the production of IL-1β from BMMOs. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with either heat-killed *S. uberis* strains 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO and/or 0.002 µM rSUB1154 or rSUB1154NP protein. Supernatants were collected 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD. Data was assumed to be normally distributed as the N was too small for a normality test to be completed, so was statistically analysed with a one-way ANOVA followed by Tukey multiple comparisons post hoc test (****P<0.0001).

3.3.5. SUB1154 primes the inflammasome; transcriptional changes

To understand where in the BMMO inflammasomal pathway SUB1154 functions, RNA was extracted from BMMOs following challenge with LPS, *S. uberis* stains 0140J or 0140JΔ*sub1154*, rSUB1154 or rSUB1154NP. RNA transcriptional changes were determined for the target genes TLR2, NF-κB, pro-IL-1β, pro-IL-18, NLRP3, pro-caspase-1 and NEK7 by real time reverse transcription quantitative PCR (Fig 3.10).

Little or no increased transcription of TLR2 was detected following treatment with *S. uberis* 0140JΔ*sub1154* and rSUB1154/NP. Stimulation with *S. uberis* strain 0140J increased transcription of TLR2 at 2h, this returned to baseline between 4-12h and showed a small increase again at 16h that decreased by 20h. Transcription of TLR2 showed a consistently elevated response following treatment of BMMOs with LPS.

Broadly, LPS and *S. uberis* strain 0140J induced similar changes in measured mRNAs, with increased NF-κB transcription at 2h returning to baseline by 4h. This was followed by increased mRNA abundance at 8h that gradually returned to baseline transcription at 20h. The *S. uberis* SUB1154 deletion mutant (0140JΔ*sub1154*) initially induced similar NF-κB transcription as *S. uberis* strain 0140J, however, there was no return to baseline at 4h and instead mRNA peaked at 2h before gradually decreasing to baseline transcription at 20h. On the other hand, treatment with rSUB1154/NP induced double the transcription of NF-κB compared to *S. uberis* strains 0140J and 0140JΔ*sub1154* treatment at 2h. This transcription level persisted from 2h to 16h before decreasing to near baseline transcription at 20h.

Treatment with *S. uberis* strain 0140J induced transcription of pro-IL-1β with an increase of $\log_2 3$ by 4h which then steadily decreased until 12h. This was followed by an increase in pro-IL-1β transcription that peaked at $\log_2 5$ by 20h. Treatment with the *S. uberis* SUB1154 deletion mutant (0140JΔ*sub1154*) did not alter pro-IL-1β transcription, which remained at baseline for the duration. Stimulation with rSUB1154/NP induced an increase in pro-IL-1β transcription to peak at $\log_2 5$ by 2h, which returned to baseline transcription by 8h; absent of the biphasic induction of pro-IL-1β induced by incubation with *S. uberis* strain 0140J.

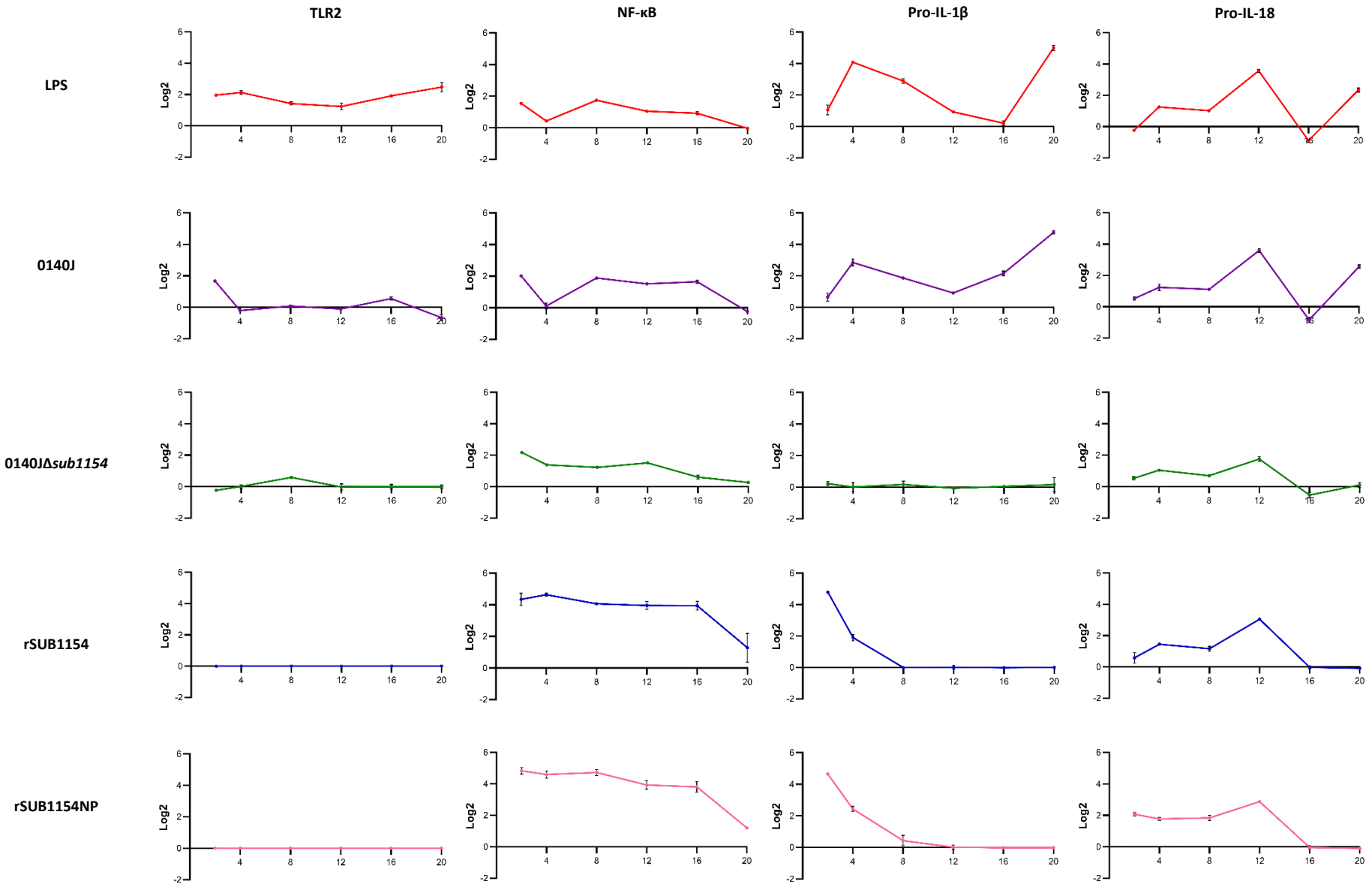
BMMOs challenged with *S. uberis* strains 0140J, 0140JΔ*sub1154*, rSUB1154 and rSUB1154NP all induced increased pro-IL-18 mRNA abundance by 4h, which remained at the same level by 8h. All stimulants resulted in a peak in pro-IL-18 mRNA at 12h; the extent of which varied with BMMOs challenged with *S. uberis* strain 0140J reaching $\log_2 4$, rSUB1154/NP $\log_2 3$ and the SUB1154 deletion mutant (0140JΔ*sub1154*) $\log_2 2$. At 16h, pro-IL-18 mRNA abundance

returned to baseline, followed by an increase to $log_2$3 only in BMMOs treated with *S. uberis* strain 0140J.

Stimulation of BMMOs with *S. uberis* strains 0140J and 0140JΔ*sub1154* induced a small increase in NLRP3 mRNA abundance at 2h, which returned and remained at baseline until 12h, followed by a gradual decrease in NLRP3 mRNA abundance to log_2-2 at 20h. However, treatment with rSUB1154/NP resulted in NLRP3 mRNA abundance constantly below baseline transcription, slightly fluctuating around log_2-2 over the time course.

S. uberis strains 0140J, 0140JΔ*sub1154* and rSUB1154/NP induced similar transcription of pro-caspase-1. There was a peak in transcription at 2h which returned to baseline by 4h. Afterwards, baseline transcription was maintained in the presence of rSUB1154/NP. Treatment with *S. uberis* strains 0104J and 0140JΔ*sub1154* decreased pro-caspase-1 transcription to log_2-1 at 8h, which was maintained throughout the study for *S. uberis* strain 0140J treatment. There was a further decrease in pro-caspase-1 transcription with the *S. uberis* SUB1154 deletion mutant (0140JΔ*sub1154*) treatment to log_2-2.5 by 16h.

Challenge of BMMOs with *S. uberis* strains 0140J and 0140JΔ*sub1154* resulted in an initial increase in NEK7 mRNA abundance with a $log_2$1 which then decreased to baseline transcription and remained for *S. uberis* 0140JΔ*sub1154* treatment. rSUB1154/NP induced below baseline transcription of NEK7 mRNA, which consisted at log_2-2 throughout. Conversely, NEK7 mRNA abundance decreased from log_2-1 at 12h to log_2-7 at 20h in BMMOs challenged with *S. uberis* strain 0140J.


TLR2
NF-κB
Pro-IL-1β
Pro-IL-18
LPS
0140J
0140JΔsub1154
rSUB1154
rSUB1154NP
Log2

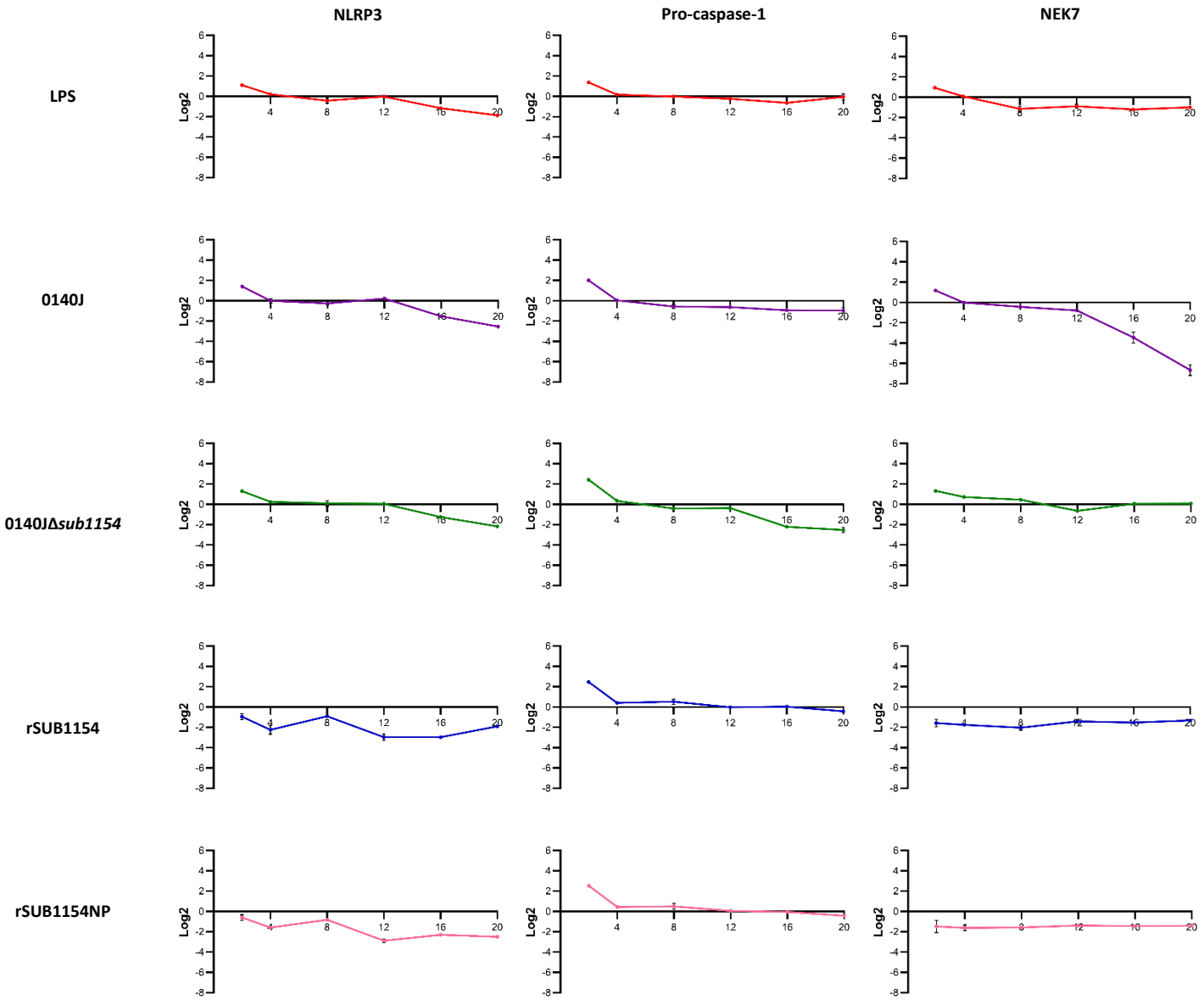


Fig 3.10. BMMO differential mRNA abundance of inflammasome pathway genes in response to *S. uberis* and SUB1154 stimulation. RNA was extracted from isolated bovine mammary macrophages (BMMOs) at 0, 2, 4, 8, 12, 16 and 20h after challenge with either LPS (10 ng/mL); heat-killed *S. uberis* strains 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO; 0.002 µM rSUB1154 or rSUB1154NP (proteolytically compromised) protein. RNA transcription changes were determined by real time reverse transcription quantitative PCR using 3 reference genes (ACTB, actin beta; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPL13a, ribosomal protein) for 7 target genes (TLR2, toll-like receptor 2; NF-κB, nuclear factor kappa B; pro-IL-1β, interleukin-1 beta precursor; pro-IL-18, interleukin-18 precursor; NLRP3, nod-like receptor (NLR) family pyrin domain containing 3; pro-caspase-1; NEK7, never in mitosis gene A (NIMA)-related kinase 7). Log2 was calculated and the data is presented as N=3±SD. Values at 0h were used to determine baseline transcription (0 Log_2).

3.3.6. SUB1154 does not affect caspase-1 activity

To determine whether SUB1154 influences caspase-1 activity, BMMOs were lysed after challenge with *S. uberis* strain 0140J, 0140JΔ*sub1154*, rSUB1154 or rSUB1154NP and a caspase-1 activity assay was performed (Fig 3.11). Baseline caspase-1 activity in unstimulated BMMOs remained at ~0.004 units. BMMOs challenged with LPS induced persistent caspase-1 activity that peaked at 0.012 units by 2h and gradually decreased to 0.008 units by 20h. Treatment with both *S. uberis* strain 0140J and 0140JΔ*sub1154* stimulated caspase-1 activity, starting at 0.007 units by 2h, peaking at 0.009 units by 12h, followed by a decrease in activity returning to 0.007 units by 20h. Caspase-1 activity in BMMOs challenged with rSUB1154 also showed some elevation (0.007 units) by 2h and peaked (0.011 units) by 4h. The SUB1154 protein does not change caspase-1 activity in the presence of *S. uberis* bacterial cells.

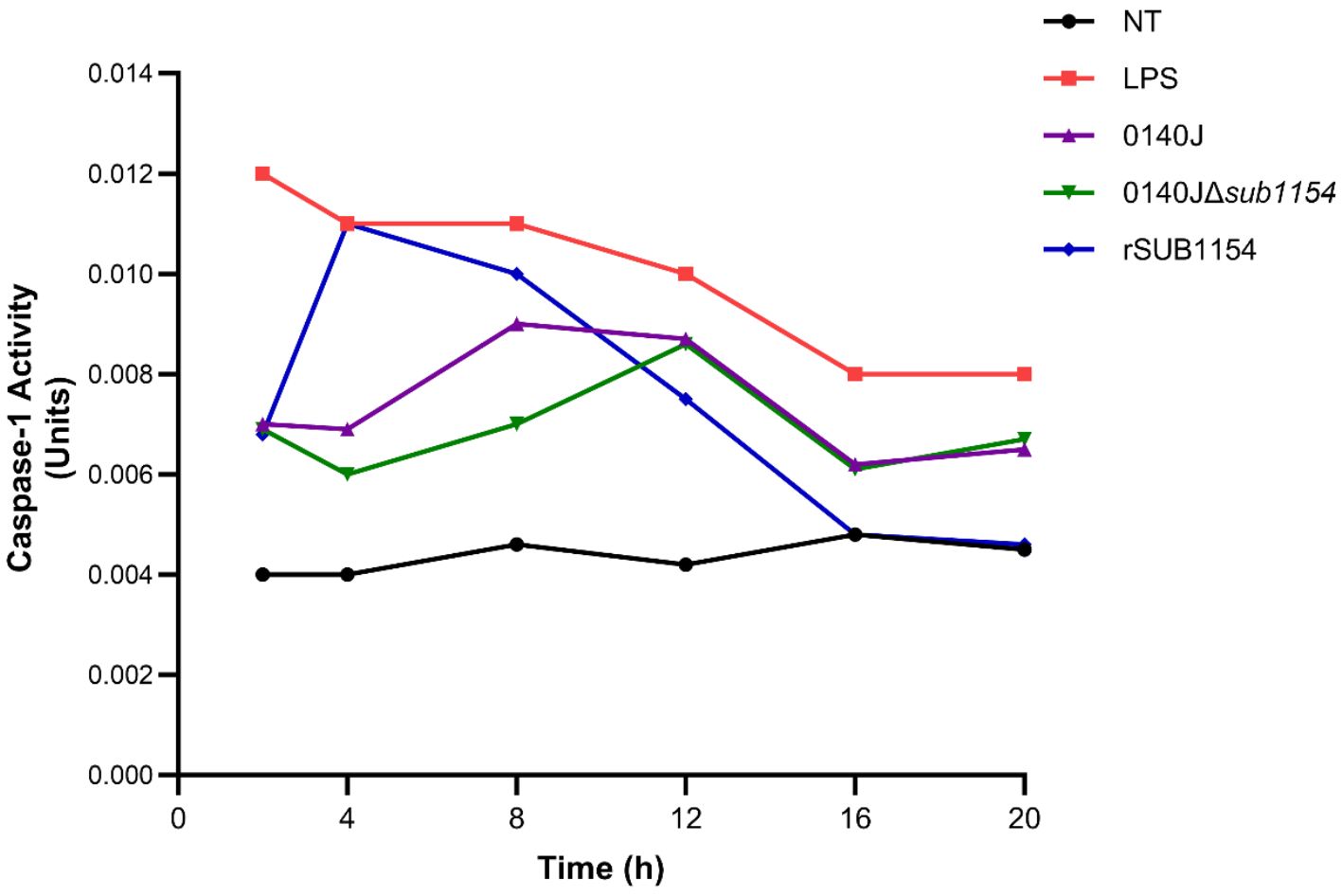


Fig 3.11. Caspase-1 activity is not dependent on SUB1154. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were either unstimulated (NT) or challenged with LPS (10 ng/mL); heat-killed *S. uberis* strain 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO or 0.002 µM recombinant SUB1154 protein. Caspase-1 activity was determined 2, 4, 8, 12, 16 and 20h after challenge by measuring colorimetric values at 400 nm and units generated by interpolating using a standard curve. Data is presented as N=3.

3.3.7. SUB1154 primes the inflammasome; manipulation of priming and activation signals

To determine how SUB1154 interacts with the NLRP3 inflammasomal pathway, the ability to prime and activate the inflammasome separately was needed. Pam3CSK4 can prime the NLRP3 inflammasome but not activate it whilst silica activates the NLRP3 inflammasome but does not prime it. This needed to be confirmed in the setting of BMMOs.

The NLRP3 inflammasome in BMMOs was primed by treatment with varying concentrations of Pam3CSK4, and the activation signal was provided by stimulation with silica (Fig 3.12). BMMOs stimulated with 1000 μg/mL silica alone secreted significantly more IL-1β compared to challenge with 500 μg/mL silica ($P<0.05$). Therefore, 500 μg/mL silica was used for subsequent experiments. BMMOs stimulated with Pam3CSK4 (priming signal alone) secreted minimal IL-1β, with no significant difference between 0.5, 1.0 and 2.0 μg/mL Pam3CSK4. With the addition of the activation signal, silica (500 μg/mL), there was significantly more IL-1β secreted from BMMOs stimulated with 1.0 μg/mL Pam3CSK4 compared to 0.5 μg/mL Pam3CSK4 ($P<0.0001$). Therefore, 1.0 μg/mL of Pam3CSK4 was used for future experiments.

As in the preliminary experiment (above and Fig 3.12), neither BMMO treatment with Pam3CSK4 nor silica alone resulted in significant production of IL-1β. BMMOs challenged with *S. uberis* SUB1154 deletion mutant (0140JΔ*sub1154*) and the inflammasome primer Pam3CSK4 induced production of IL-1β (50% of that obtained following treatment with the LPS control), but no significant level of IL-1β was detected when BMMOs were challenged with *S. uberis* 0140JΔ*sub1154* in combination with the inflammasome activator silica (Fig 3.13). Conversely, BMMOs challenged with rSUB1154 or rSUB1154NP and Pam3CSK4 produced minimal levels of IL-1β, but when challenged with either recombinant protein in combination with silica BMMOs produced significant levels of IL-1β (~30% of that obtained following stimulation with LPS). Cumulatively, these data suggest that SUB1154 primes but does not activate the inflammasome. Interestingly, BMMOs activated with silica and rSUB1154NP produced significantly less IL-1β compared to those challenged with silica and rSUB1154 ($P<0.01$).

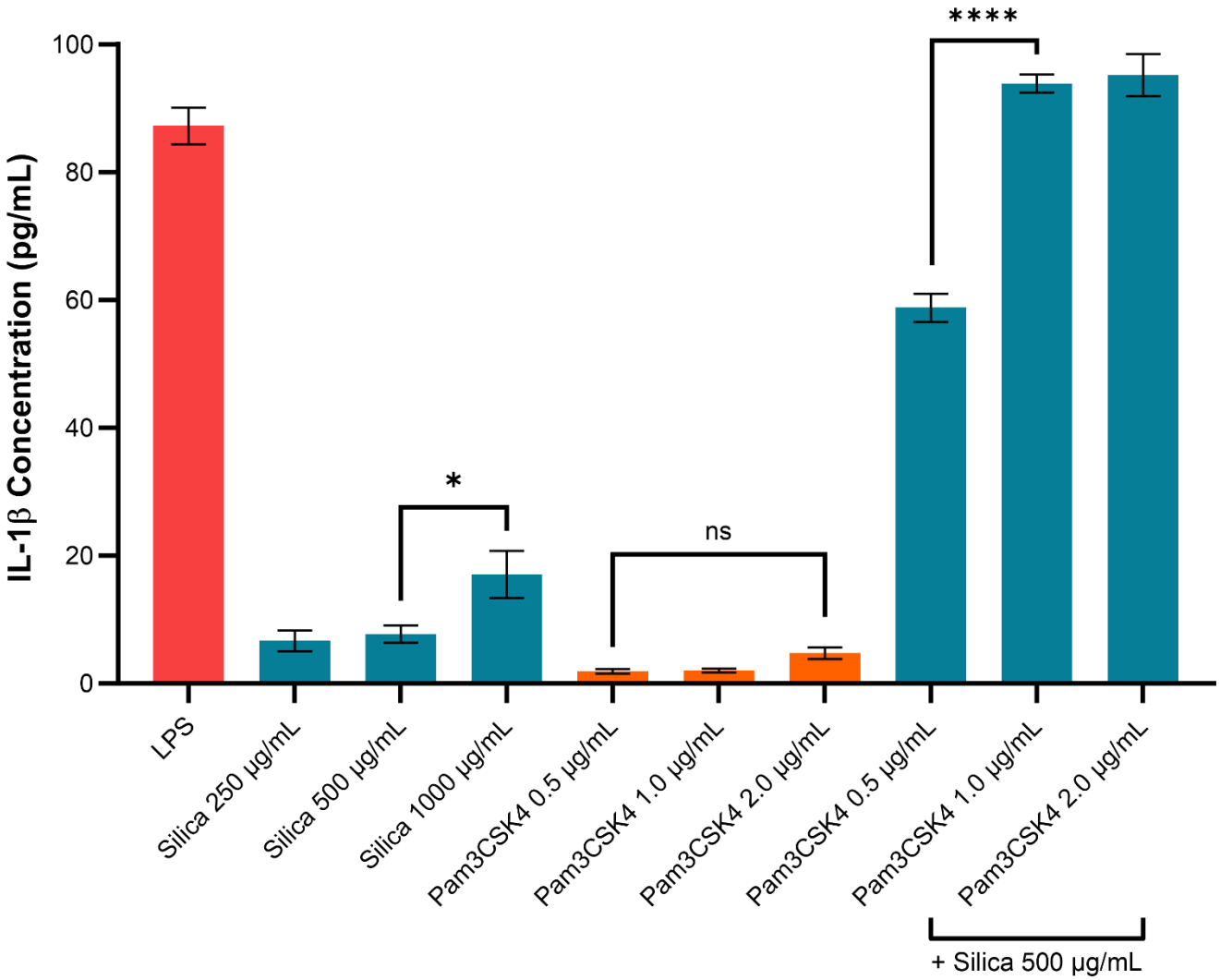


Fig 3.12. Optimal BMMO challenge concentrations of Pam3CSK4 and silica. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with LPS (10 ng/mL); NLRP3 inflammasome activator silica (250, 500 or 1000 µg/mL) or NLRP3 inflammasome primer Pam3CSK4 (0.5, 1.0 or 2.0 µg/mL). Supernatants were collected at 20h after challenge and the concentration of IL-1β was measured by ELISA. Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Tukey multiple comparison post hoc test (*P<0.05; ****P<0.0001; ns = not significant).

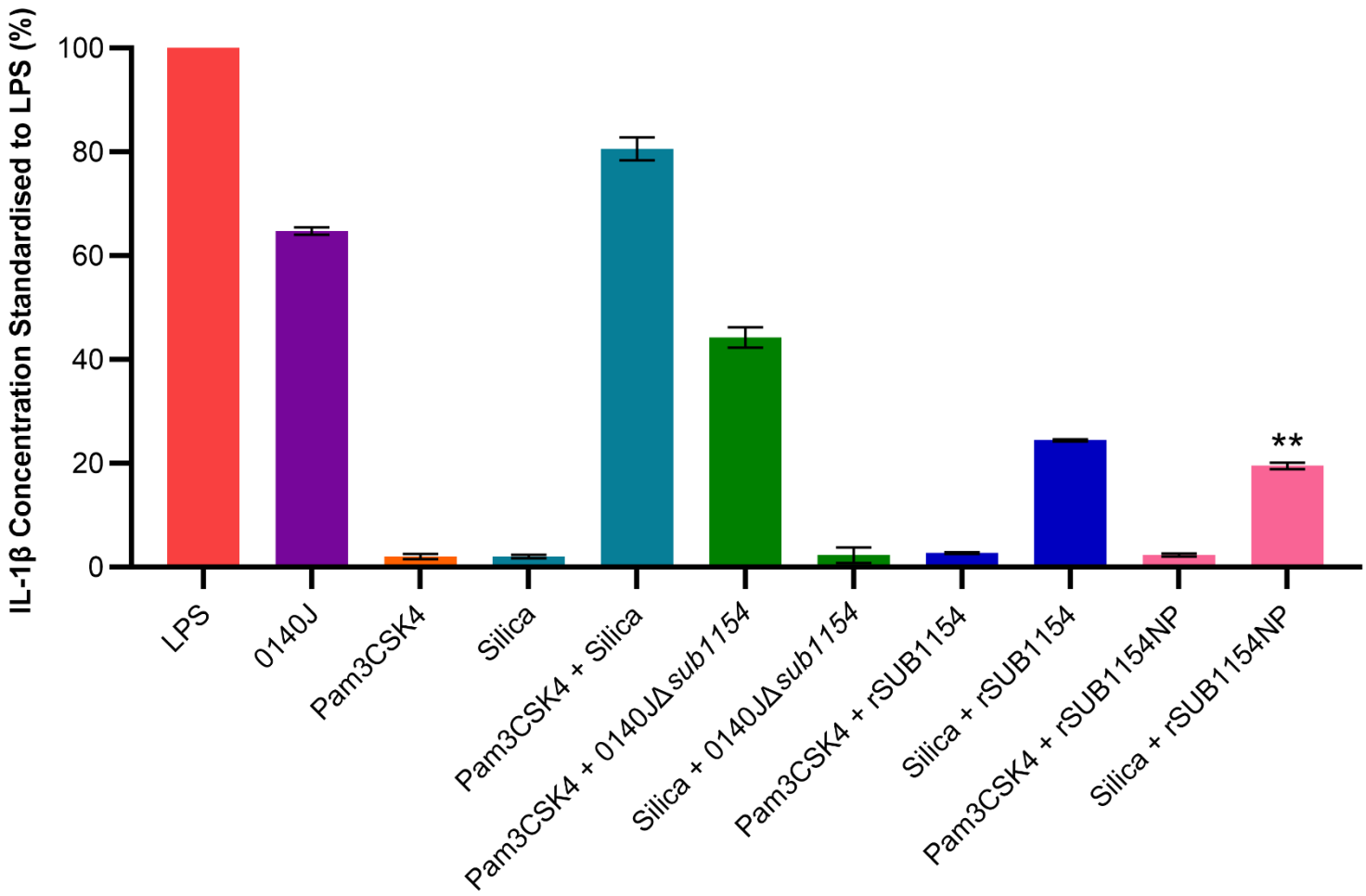


Fig 3.13. *S. uberis* SUB1154 protein primes the NLRP3 inflammasome in BMMOs. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO; 0.002 µM rSUB1154 or rSUB1154NP (proteolytically compromised) protein; Pam3CSK4 (1.0 µg/mL) and/or silica (500 µg/mL). Supernatants were collected at 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Tukey multiple comparisons post hoc test (**P<0.01 compared to silica + rSUB1154).

3.3.8. SUB1154 primes the NLRP3 inflammasome intracellularly

Thus far, my data shows that SUB1154 primes the NLRP3 inflammasomal pathway leading to IL-1β secretion. However, it has yet to be confirmed whether SUB1154 functions extracellularly or intracellularly. IL-1β secretion was measured from BMMOs following challenge with *S. uberis* strain 0140J in the presence and absence of the cell entry inhibitor, Cytochalasin D (CyD) (Fig 3.14). In the presence of CyD, where *S. uberis* strain 0140J was inhibited from entering the BMMOs, there was ablation of the IL-1β response. BMMOs were challenged with *S. uberis* 0140JΔ*sub1154*, allowing time for the bacteria to enter the BMMOs. This was followed by incubation with CyD and subsequently rSUB1154, which was now inhibited from entering the BMMOs. Ultimately, this resulted in no IL-1β production from BMMOs. These data suggest that SUB1154 functions intracellularly.

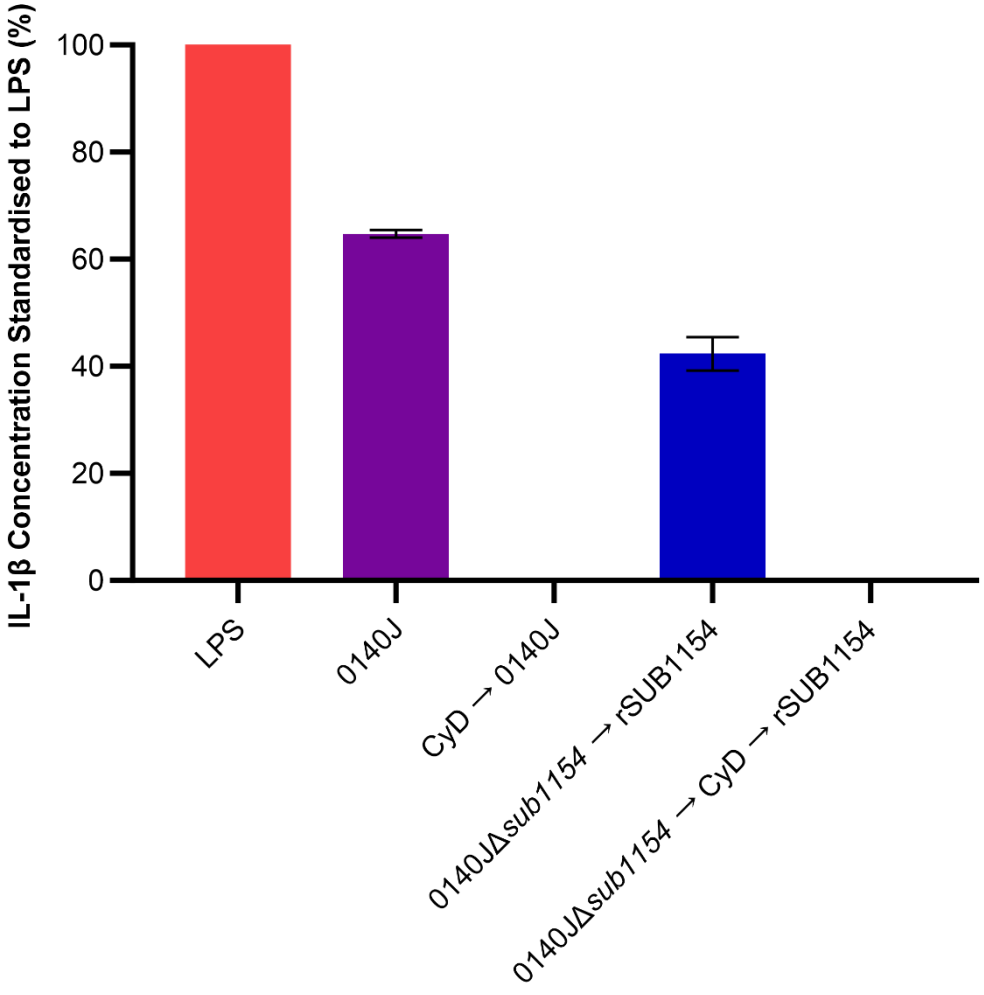


Fig 3.14. *S. uberis* SUB1154 protein primes the NLRP3 inflammasome intracellularly. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO and/or 0.002 µM rSUB1154 protein. Cell entry was inhibited by incubating BMMOs with Cytochalasin D (Cyd; 10 µM) for 2h. Supernatants were collected at 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD.

3.3.9. TLR2 is required for SUB1154 priming of the BMMO NLRP3 inflammasome

As TLR2 is a common PRR for detection of Gram-positive bacteria, next we sought to investigate its role in the production of IL-1β (Fig 3.15). Prior to BMMO challenge, extracellular binding to TLR2 was inhibited by the antagonist MMG 11. Alternatively, the intracellular interactions between TLR2-TIR domain and the downstream signalling cascade were inhibited by C29. MMG 11 failed to inhibit production of IL-1β, however, C29 completely abolished IL-1β production from BMMOs stimulated with SUB1154 (in the context of *S. uberis* strain 0140J, rSUB1154 and rSUB1154NP).

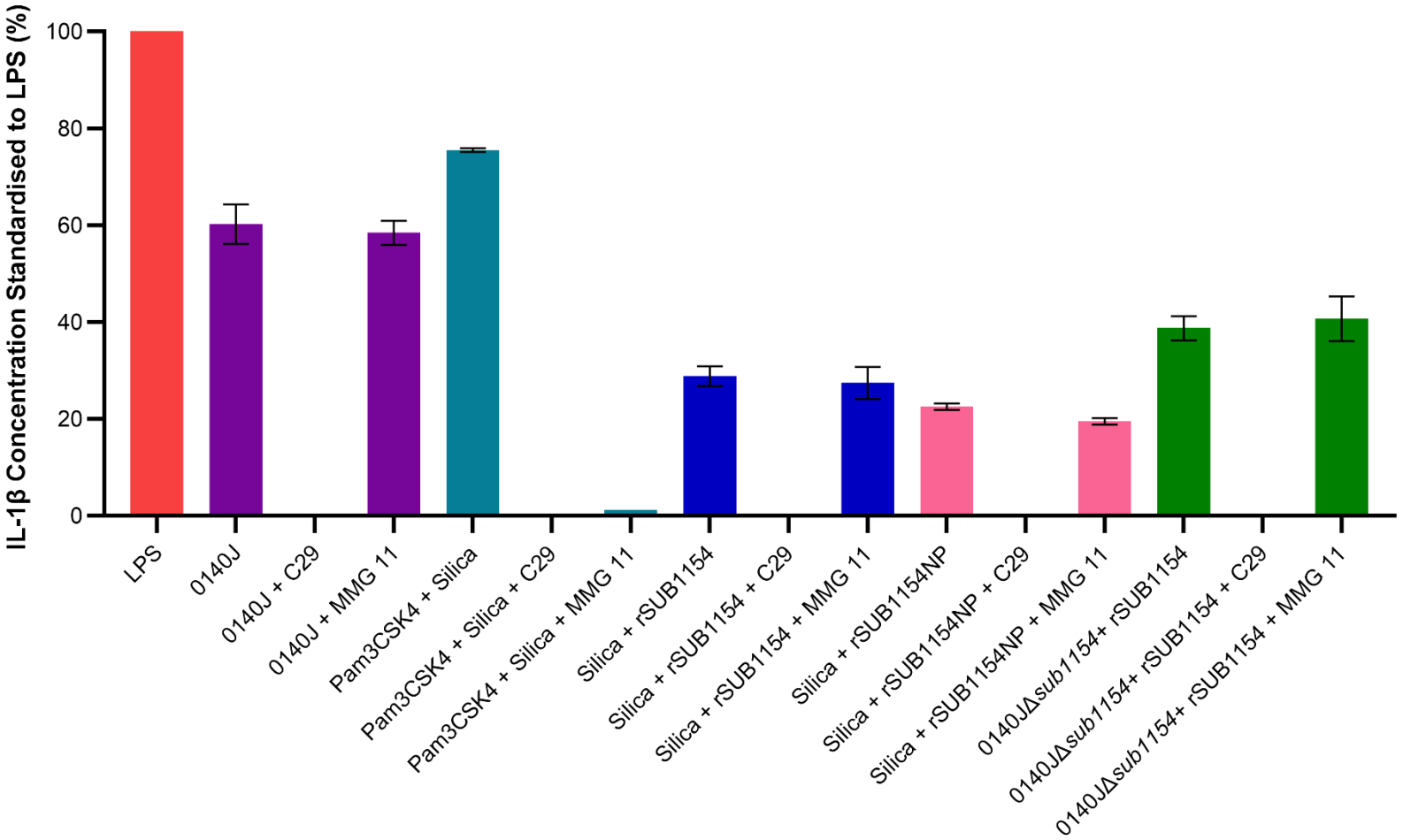


Fig 3.15. TLR2 is involved in SUB1154 priming of the NLRP3 inflammasome. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were incubated with either MMG 11 (100 µM; antagonist to extracellular binding of TLR2) or C29 (100 µM; intracellularly inhibits TLR2-TIR (toll-interleukin receptor) domain) for 1h prior to subsequent challenge with heat-killed *S. uberis* strain 0140J or SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO; 0.002 µM rSUB1154 or rSUB1154NP (proteolytically compromised) protein; Pam3CSK4 (1.0 µg/mL; primes the inflammasome) and/or silica (500 µg/mL; activates the inflammasome). Supernatants were collected at 20h after challenge and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD.

3.4. Discussion

The *S. uberis* SUB1154 protein has been suggested to be involved in the BMMO inflammasomal pathway resulting in the production of the pro-inflammatory cytokine IL-1β (Archer *et al*., 2020). However, the precise mechanism of action of SUB1154 has yet to be determined. Therefore, it is important to investigate how SUB1154 results in IL-1β production in order to understand the initial interactions between *S. uberis* and BMMOs.

3.4.1. S. uberis causes IL-1β production from BMMOs via the NLRP3 inflammasome and caspase-1

In line with previous studies, results in this study confirmed that IL-1β production from BMMOs stimulated with *S. uberis* is dependent on the NLRP3 inflammasome (Archer *et al*., 2020). Incubation of BMMOs with three different inflammasome inhibitors prior to challenge with *S. uberis* all resulted in ablation of IL-1β production. MCC950 is a potent and specific NLRP3 inhibitor that binds proximal to the Walker B motif within the NACHT domain of NLRP3 in both its inactive and active conformation (Fig 3.16). This prevents NLRP3 ATP hydrolysis, inhibiting inflammasome assembly and function. The NACHT domain of NLRP12 is the most closely related to the NACHT domain of NLRP3, however, MCC950 was found to not inhibit the chimeric form of NLRP3 in which the NACHT domain was swapped for that of NLRP12 (Coll *et al*., 2019). Therefore, this confirms that *S. uberis* activates the NLRP3 inflammasome in BMMOs and no other inflammasomal complexes appear to be involved.

Caspases are proteolytic enzymes that can be divided into two groups based on their role in inflammation and apoptosis. There are five caspases that have been termed inflammatory following their identification as mediators in the innate immune response. These include human caspase-1, -4, -5, -12 and mouse caspase-1, -11 and -12 (Eckhart *et al*., 2008; Shalini *et al*., 2015). Pro-IL-1β is cleaved into its active form by capase-1 in regard to the NLRP3 inflammasome during *S. pyogenes* infection (Harder *et al*., 2009). Caspase-4, -5 and -11 function by directly binding to cytoplasmic LPS causing oligomerisation and cell death (Eckhart *et al*., 2008; Shalini *et al*., 2015). Caspase-13 was discovered as unique to bovine cells and has been described as an orthologue to human caspase-4 (Humke *et al*., 1998; Koenig *et al*., 2001). The inhibitors AC-YVAD-CMK and Z-YVAD-FMK both inhibit caspase-1 activity in addition to caspase-4 and caspase-3, respectively (Fig 3.16). Ablation of IL-1β in BMMOs treated with either of these inhibitors prior to *S. uberis* stimulation suggest that pro-IL-1β is cleaved by caspase-1.

3.4.2. SUB1154 is essential in the production of IL-1β from BMMOs challenged with S. uberis

Previous research has shown that the SUB1154 protein elicits an inflammatory response to *S. uberis* in BMMOs (Archer *et al*., 2020). BMMOs stimulated with 20, 10 and 5 µM purified SUB1154 protein alone resulted in IL-1β production at relatively low concentrations. However, no IL-1β was produced when BMMOs were challenged with 2.5, 2, 0.2, 0.02 and 0.002 µM. The response observed at higher SUB1154 concentrations was most likely by the overstimulation of BMMOs due to the extremely high concentration of protein and therefore any protein present at such levels would cause an IL-1β response. Archer *et al*., 2020, stimulated BMMOs with a final concentration of 0.02 µM recombinant SUB1154 protein. Due to no IL-1β production below challenge with 2.5 µM it can therefore be concluded that SUB1154 protein alone does not induce IL-1β production in BMMOs. Therefore, subsequent experiments challenged with 0.002 µM SUB1154 protein to correspond a similar concentration of SUB1154 molecules present when BMMOs are challenged with the *S. uberis* strain 0140J.

Evaluation of the response of *S. uberis* in the absence of SUB1154 was also conducted through the utilisation of the deletion mutant, 0140JΔ*sub1154*. Confirmation of SUB1154 deletion was determined by PCR, gel electrophoresis and lack of expression by ELISA (Chapter 4). Both *S. uberis* strain 0140J and 0140JΔ*sub1154* contained the *sub0888* gene, which is considered unique to *S. uberis*. SUB1154 was amplified and found within *S. uberis* strain 0140J but not 0140JΔ*sub1154*, confirming the deletion. Determining the function of SUB1154 was permitted through comparisons between purified SUB1154 protein, *S. uberis* strain 0140J and the deletion mutant.

Absence of IL-1β production from BMMOs stimulated with the SUB1154 deletion mutant (0140JΔ*sub1154*) compared to *S. uberis* strain 0140J indicates that the SUB1154 protein plays an important role in stimulating the BMMO NLRP3 inflammasomal pathway. However, challenge with either rSUB1154 or rSUB1154NP (proteolytically compromised) proteins alone did not result in production of IL-1β when used at more physiologically relevant (but still high) concentrations. Only when there was combined stimulation with the deletion mutant and SUB1154 protein was there restoration of IL-1β production from BMMOs. This suggests that SUB1154 must play an essential role and act as either a priming signal, initiating transcription of *nlrp3* and/or *pro-il-1β*, or an activation signal, to promote inflammasome assembly and activity and that the complementary activity is provided by another factor within the *S. uberis* bacterial cell. Challenge of BMMOs with non-protease SUB1154 resulted in a significant reduction, but not ablation, of IL-1β production compared to treatment with intact SUB1154

protease. This suggests that SUB1154 is not wholly dependent on its putative protease function to stimulate IL-1β production.

3.4.3. SUB1154 primes the BMMO NLRP3 inflammasome

Previous studies have shown that bacterial surface proteins perform as NLRP3 inflammasome activation signals. M protein in Group A *Streptococcus* (GAS) was reported to be released from bacterial cells and served as a caspase-1 dependent NLRP3 inflammasome activation signal, inducing the maturation and release of IL-1β from macrophages (Valderrama *et al*., 2017). In the context of *S. uberis*, transcriptomic changes detected in *ex vivo* BMMOs suggested that the SUB1154 protein activates the inflammasome in a transcriptionally independent manner (Archer *et al*., 2020). However, my data contradicts these findings and shows that SUB1154 acts as an NLRP3 inflammasome priming signal.

Silica only provides the activating signal to the NLRP3 inflammasome. This is because silica causes lysosomal degradation increasing cathepsin B in the cytosol, which in turn interacts with NLRP3, promoting cleavage of pro-capsase-1 into its active form (Chevriaux *et al.,* 2020) (Fig 3.16). Therefore, if NLRP3 is not primed, pro-caspase-1 is not in close proximity and so the inflammasomal complex cannot form, preventing pro-caspase-1 cleavage and activation. With no active caspase-1, pro-IL-1β cannot be cleaved and so explains the absence of IL-1β secreted when macrophages are challenged with silica alone. The increase in IL-1β production from BMMOs stimulated with 1000 µg/mL of silica could imply that overexposure of silica causes cell stress resulting in inflammasome priming.

Alternatively, Pam3CSK4 provides only the priming signal to the inflammasome. This is because Pam3CSK4 is a TLR2 agonist triggering downstream signalling that ultimately results in NLRP3 priming (Fig 3.16). After Pam3CSK4 priming, in the absence of the inflammasome activation signal from silica, there was minimal IL-1β production. BMMOs treated with both Pam3CSK4 (1.0 and 2.0 µg/mL) and silica resulted in IL-1β production from BMMOs reaching similar levels as when stimulated with LPS.

IL-1β production was induced from BMMOs challenged with *S. uberis* SUB1154 deletion mutant (0140JΔ*sub1154*) and Pam3CSK4 but not when stimulated with *S uberis* 0140JΔ*sub1154* and silica. Conversely, IL-1β production was not induced from BMMOs challenged with rSUB1154 and Pam3CSK4 but was when stimulated with rSUB1154 and silica. Together, these data suggest that rSUB1154 provides the BMMO NLRP3 inflammasome priming signal and another factor within the *S. uberis* bacterial cell or cellular response to the bacterial cell generates the activation signal.

SUB1154 acting as a priming signal coincides with the pro-IL-1β transcriptional data. NLRP3 inflammasomal priming induces pro-IL-1β and pro-IL-18 transcription and inflammasomal activation results in the cleavage of these cytokines into their active forms by caspase-1. BMMOs stimulated with *S. uberis* strain 0140J induced increased pro-IL-1β transcription by 4h, which then returned to baseline by 12h. This was then followed by a subsequent increase in pro-IL-1β transcription that peaked by 20h. These changes in pro-IL-1β transcription correspond with the production of active IL-1β. On the other hand, BMMOs challenged with *S. uberis* 0140JΔ*sub1154* did not induce pro-IL-1β transcription. A lack of IL-1β transcription explains the absence of IL-1β production from BMMOs challenged with *S. uberis* 0140JΔ*sub1154*. Therefore, SUB1154 primes the NLRP3 inflammasome by inducing pro-IL-1β transcription.

In contrast, there was an initial increase in pro-IL-1β transcription up to 8h when BMMOs were challenged with either rSUB1154 or rSUB1154NP, however, there was no production of active IL-1β from BMMOs stimulated with either rSUB1154 proteins alone. This raised the questions as to whether the absence of IL-1β production from BMMOs stimulated with rSUB1154, despite transcription of pro-IL-1β, was due to a lack of an NLRP3 activation signal promoting caspase-1 cleavage of pro-IL-1β or whether rSUB1154 also inhibited caspase-1 activity. Caspase-1 activity was found to be unaffected by the presence or absence of rSUB1154. These data further imply that SUB1154 provides the priming signal and another factor within the bacterial cell provides the activation signal. Pro-IL-1β transcription remains at baseline levels 8h after BMMOs are challenged with rSUB1154/NP. As there is no production of active IL-1β from BMMOs, there is a no binding of IL-1β to IL-1R (interleukin-1 receptor), preventing the second peak of pro-IL-1β transcription at 20h. Interestingly, BMMOs challenged with *S. uberis* 0140JΔ*sub1154* induced increased transcription of pro-IL-18, despite its transcription being induced via the same pathway as pro-IL-1β. Unlike pro-IL-1β, pro-IL-18 is constitutively expressed in macrophages and transcription can occur independent of NF-κB activation (Kaplanski, 2018).

As well as NF-κB functioning as a transcription factor during NLRP3 inflammasome priming, NF-κB also negatively regulates activation of the NLRP3 inflammasome. NF-κB activation in primed macrophages upregulates p62 which is recruited to damaged mitochondria to eliminate inflammasome activation signals (Zhong *et al*., 2016). NF-κB transcription is upregulated in BMMOs stimulated with either rSUB1154/NP compared to challenge with *S. uberis* 0140J or 0140JΔ*sub1154*. As there is no activation signal providing the mitochondrial

damage in BMMOs stimulated with SUB1154 protein only, p62 is unable to function and NF-κB transcription is not negatively regulated.

NLRP3 inflammasome activation is also regulated by growth factor independence 1 (Gfi1). NF-κB activation induces expression of Gfi1 which directly binds to either the Gli-responsive element 1 (GRE1) within the *nlrp3* promoter or to NF-κB, preventing NLRP3 transcription. This feedback loop tightly controls NLRP3 expression in order to prevent excessive production of IL-1β which would be damaging to the host (Zhu *et al*., 2014). NLRP3 transcription is downregulated in BMMOs stimulated with either rSUB1154/NP compared to challenge with *S. uberis* 0140J or 0140JΔ*sub1154*. Due to the lack of activation signal, in combination with high levels of NF-κB mRNA, these BMMOs have an abundance of NLRP3 that is not being activated and so NLRP3 transcription is downregulated.

These data conclude that SUB1154 provides the NLRP3 inflammasome priming signal, but the factors associated with the bacterial cell that provide the activation signal remain unknown. Inflammasome activation signals can result in potassium efflux from macrophages resulting in NEK7 dependent NLRP3 oligomerisation (Muñoz-Planillo *et al*., 2013). Interestingly, NEK7 transcription was substantially decreased in BMMOs 12h after challenge with *S. uberis* strain 0140J. Schmacke *et al*., 2019, found that NEK7 was dispensable for NLRP3 inflammasome formation under pro-inflammatory conditions. While murine macrophages were found to predominately rely on NEK7, human myeloid cell lines and primary macrophages were found to utilise TAK-1 for NLRP3 inflammasome assembly. Human macrophages were found to employ NEK7 initially, however, after prolonged stimulation, the importance of NEK7-dependent activation declined and macrophages switched to using TAK-1 instead. This possibly explains the initial increase in NEK7 transcription, followed by the rapid decline, in BMMOs stimulated with *S. uberis* strain 0140J. There was no decrease in NEK7 transcription following BMMO challenged with *S. uberis* 0140JΔ*sub1154*, because an absence of SUB1154 and hence no priming signal, means TAK-1 is not activated to promote the switch. Decrease in NEK7 transcription however was not observed in LPS stimulated BMMOs. LPS signalling occurs through TLR4. In contrast to TLR2, TLR4 signalling can additionally activate TAK1 through utilisation of the TRIF and TRAM adaptors (Kawasaki & Kawai, 2014).

3.4.4. SUB1154 interacts with BMMO TIR domains intracellularly

Following the establishment that SUB1154 primes the inflammasome, the mechanism by which this occurs needed elucidation. In the context of bacteria, the priming signal is normally presented through TLR signalling (Gong *et al*., 2020; Duez & Pourcet, 2021). However, previous

studies have indicated that *S. uberis* does not interact with TLR2 expressed on bMECs but does activate NF-κB (downstream of the TLR2 signalling pathway) (Günther *et al.,* 2016a). This coincides with the increase in NF-κB transcription, but no such increase in TLR2 transcription following BMMO stimulation with *S. uberis* strain 0140J. To investigate whether this was the also the case for BMMOs, TLR2 binding was inhibited prior to challenge with *S. uberis* strain 0140J.

MMG 11 acts as a competitive antagonist that displaces Pam3CSK4 (Grabowski *et al*., 2018 & 2020) (Fig 3.16). Pam3CSK4 is a triacylated lipopeptide ligand that bridges the two TLR N-terminal ectodomains by inserting two ester-bound palmitoyl groups into the TLR2 binding pocket and the single amide-bound palmitoyl chain into the TLR1 pocket. The head group of Pam3CSK4 may also interact with TLR1 and TLR2 through the formation of hydrogen bonds with glycerol and hydrophobic interactions with sulphur atoms (Botos *et al*., 2011). Despite MMG 11 inhibiting both TLR2/1 and TLR2/6 signalling, MMG 11 was found to preferentially inhibit TLR2/1 (Grabowski *et al*., 2018 & 2020). No difference was found in IL-1β production from BMMOs challenged with *S. uberis* strain 0140J; silica and rSUB1154/NP or *S. uberis* 0140JΔ*sub1154* and rSUB1154 in the presence and absence of MMG 11. In line with bMECs, these data suggest that *S. uberis* does not interact with extracellular LRR domain of TLR2 expressed on BMMOs. This is advantageous to the bacteria as it evades the immune system allowing colonisation by delaying the initiation of an immune response from both bMECs and BMMOs.

The SUB1154 priming signal could therefore either be provided through interactions with other extracellular receptors or occur intracellularly. BMMOs were stimulated with *S. uberis* 0140JΔ*sub1154* prior to incubation with the cell entry inhibitor CyD; allowing time for internalisation and/or the generation of the NLRP3 inflammasome activation signal. Subsequently, after cell entry inhibition, BMMOs were challenged with rSUB1154 protein, which would remain extracellular to the BMMOs. This is because CyD inhibits actin polymerisation, which blocks >90% of macrophage endocytosis (Ribes *et al*., 2010). These conditions resulted in no IL-1β production from BMMOs. Therefore, these data suggest that the SUB1154 primes the BMMO NLRP3 inflammasome intracellularly. SUB1154 could interact with TLR2 intracellularly, or other TLRs that are expressed on endosomal membranes, such as TLR8 or 9 (Zinkernagel *et* al., 2012; Eigenbrod & Dalpke, 2015). Alternatively, CyD could have prevented the internalisation of SUB1154 bound TLR2, and thus no signal occurred.

Intracellular TLR2 signalling was inhibited using C29, which binds to the pocket within the BB loop of the TLR2-TIR domain (Fig 3.16). This inhibits TLR2-TIR interactions with TIRAP-TIR, preventing the downstream MAPK and NF-κB activation, ultimately removing the NLRP3 inflammasome priming signal (Mistry *et al*., 2015). BMMOs challenged with *S. uberis* strain 0140J; silica and rSUB1154/NP or *S. uberis* 0140JΔ*sub1154* and rSUB1154 in the presence of C29 resulted in ablation of IL-1β production. If SUB1154 provided the NLRP3 inflammasome priming signal downstream of MyD88 then it would be expected that IL-1β production would not be affected by the inhibitory effects of C29 as the MAPK pathway would still become activated. Therefore, these data suggest that SUB1154 primes the NLRP3 inflammasome through BMMO TIR domain interactions.

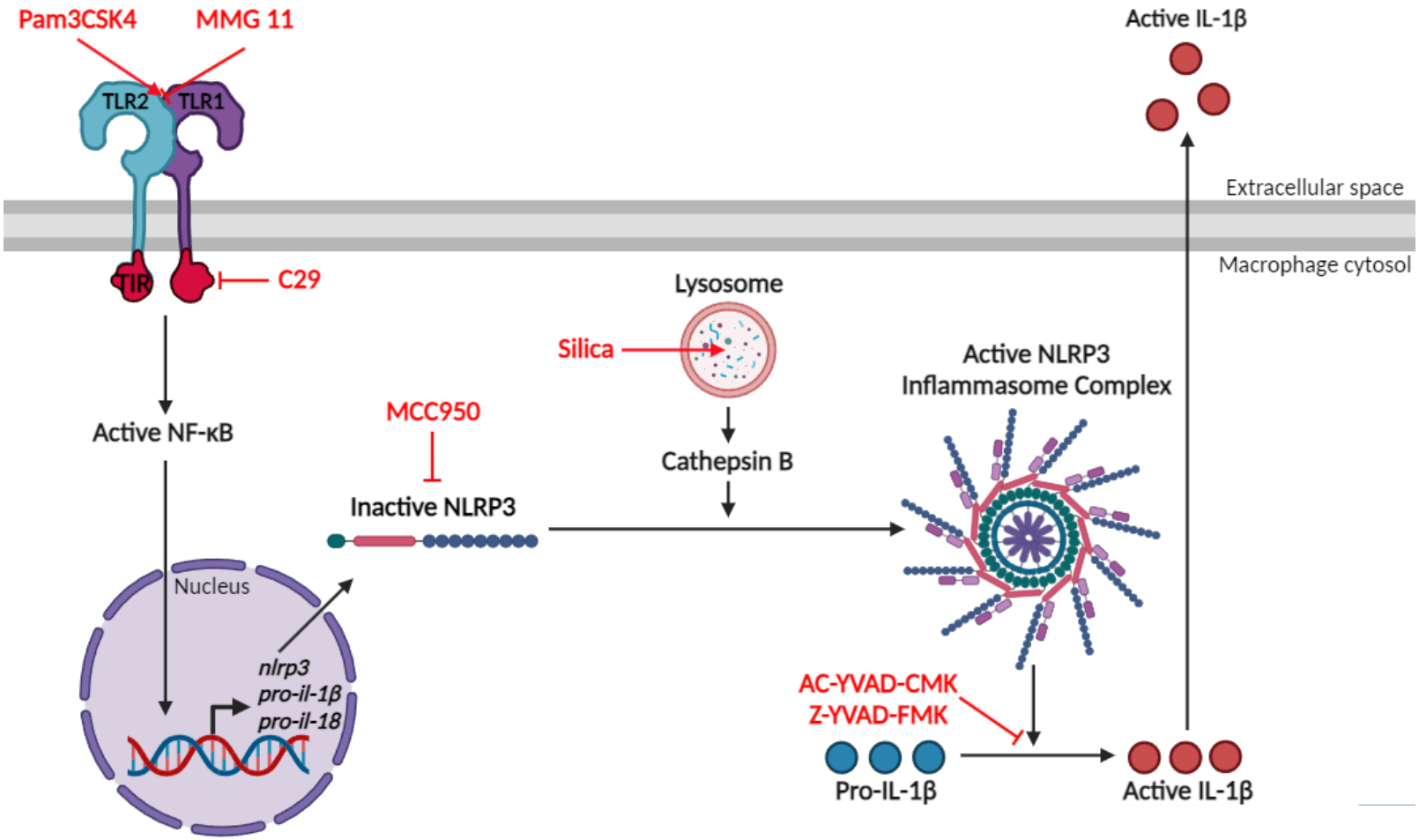


Fig 3.16. Stimulators and inhibitors in the NLRP3 inflammasomal pathway. Pam3CK4 is a triacylated lipopeptide ligand that primes the NLRP3 inflammasome by binding to the extracellular leucine rich repeat of TLR2 and TLR1 (toll-like receptor). MMG 11 acts as a competitive antagonist that displaces Pam3CSK4, preventing the TLR2 downstream signalling cascade. C29 binds to the pocket within the BB loop of the TLR2-TIR (toll-interleukin receptor) domain, preventing activation of NF-κB (nuclear factor kappa B). Silica provides the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) activation signal by causing lysosomal degradation, increasing cathepsin B in the cytosol, which in turn interacts with NLRP3, promoting cleavage of pro-caspase-1 into its active form. MCC950 is a potent and specific NLRP3 inhibitor that binds to NLRP3 preventing assembly and function of the active NLRP3 inflammasome complex. AC-YVAD-CMK and Z-YVAD-FMK inhibit caspase-1 activity (as well as caspase-4 and caspase-3 respectively) preventing cleavage of pro-IL-1β into its active form. Figure created using BioRender.com.

3.4.5. Examples of other bacterial-TIR interactions

TIR domains are composed of a central five-stranded parallel β sheet surrounded by five α helices (Patterson & Werling, 2013). TLR1 and TLR2 share 50% sequence identity with conformational differences. Each TIR domain contains both a BB loop and a DD loop. The BB loop, which joins strands βB and αB, is an essential site that contains a key proline residue that is exposed for interactions with the TIR of adaptor proteins during signal transduction (Dunne *et* al., 2003; O'Neill & Bowie, 2007; Botos *et al*., 2011). Mutations in the BB loop were found insufficient to prevent recruitment of adaptor proteins in TLR4-TIR, however, this mutation in TLR2-TIR abolished the binding of MyD88. TIR domains interact through the binding of the BB loop of one TIR domain to the DD loop of the other TIR domain (e.g., TLR2 BB loop interacts with DD loop of MyD88 and the TLR2 DD loop interacts with the BB loop of TLR1) (O'Neill & Bowie, 2007).

Bacteria possess TIR domain containing proteins (Tcps) that are thought to target mammalian TIR complexes (Lee *et al*., 2022). One example is TlpA (TIR-like protein A), which was identified in *Salmonella enterica* serovar Enteritidis to have structural homology to TIR-like domains. TlpA was found to suppress NF-κB activation while simultaneously promoting activation of caspase-1 resulting in the secretion of IL-1β from infection of cultured macrophages. This suggests that the protein may act differently during infection to either promote or suppress cytokine induction. Additionally, TlpA was found to be required for intracellular growth and survival of bacteria as there was reduced accumulation of bacteria inside infected macrophages in the absence of TlpA. Therefore, despite TlpA triggering IL-1β production that activates the immune response, TlpA is required to prevent activation of endogenous host cell pathways that suppress accumulation of intracellular bacteria (Newman *et al*., 2006).

Tcps were subsequently identified in *Escherichia coli* CFT073 (TcpC) (Cirl *et al*., 2008) and *Brucella melitensis* (TcpB) (Radhakrishnan *et al*., 2009; Sengupta *et al*., 2010). TcpC was found to impair TLR2 and TLR4 mediated activation of NF-κB by interacting with MyD88 and suppressing MyD88 signalling (but not TRIF) (Cirl *et al*., 2008). TcpC was identified as a virulence factor that increased bacterial burden and tissue damage in the mouse UTI model, promoting intracellular accumulation of bacteria (Cirl *et al*., 2008). Despite TcpC being secreted from bacterial cells, secreted TcpC, along with a purified recombinant version of the protein was found to accumulate intracellularly to inhibit host TLR signalling (Cirl *et al*., 2008). On the other hand, TcpB was found to mimic TIRAP, through binding to phosphoinositides at the

plasma membrane, and inhibit TIRAP-induced TLR2-mediated NF-κB activation through targeted degradation (Radhakrishnan *et al*., 2009; Sengupta *et al*., 2010).

Similar to TcpC, the *S. aureus* Tcp TirS is secreted from cells and exerts its TLR2-mediated NF-κB signalling inhibitory effect on the NF-κB reporter in the absence of *S. aureus* invasion into host cells. Like other bacterial Tcps, TirS is essential for increasing bacterial survival within host cells, however, can target both MyD88 and TLR2 directly (Askarian *et al*., 2014). TirS was also found to function enzymatically as an NADase that reduces NAD^+ levels in mammalian cells. Subsequently, the presence of Tcps that cleave NAD^+ in non-pathogenic bacteria suggests that these proteins may also play a role in regulating metabolic pathways in a non-virulent manner (Essuman *et al*., 2018).

These studies demonstrate bacterial-TIR interactions are not unique to *S. uberis* and have differing consequences on the inflammasomal pathway depending on the bacterial protein. Therefore, it is important to ascertain whether the SUB1154-TIR interaction benefits or hinders *S. uberis* pathogenesis. *S. uberis* SUB1154 protein has been shown to be important for colonisation, which is similar to TlpA, TcpC and TirS, which were found to be essential for bacterial growth and survival. Additionally, my data has shown that SUB1154 functions intracellularly, which was also the case for TcpC and TirS. Although many bacterial TIRs have been investigated to inhibit host TLR signalling, TlpA has also been shown to trigger IL-1β production, similar to SUB1154, in conjunction with inhibition of NF-κB activation. SUB1154 may function at the TIR domain of TLR2, TIRAP, MyD88 or IL-1R, as these are the common sites for other bacterial TIRs to form interactions. Due to both SUB1154 and TlpA inducing IL-1β production, SUB1154 may also have an additional function aside from inflammasomal priming and promoting colonisation, that inhibits the BMMO immune response.

Conclusion

These data suggest that the *S. uberis* SUB1154 protein is essential for the secretion of IL-1β from BMMOs. SUB1154 provides the NLRP3 inflammasome priming signal and another factor within the *S. uberis* bacterial cell generates the activation signal. *S. uberis* evades initiation of the bovine immune response by not interacting with TLR2 expressed on the extracellular surface of bMECs and BMMOs. Instead, SUB1154 must become internalised into the BMMO, possibly during immune surveillance, where is acts intracellularly probably through interactions with BMMO TIR domains.

Chapter 4: The SUB1154 protein between *S. uberis* strains

4.1. Introduction

4.1.1. Phagocytosis and intracellular killing of S. uberis

Macrophages and neutrophils are part of the first line of defence against invading bacterial pathogens due to their phagocytic and bactericidal capabilities. Bacteria bind to PRRs expressed on the surface of these cells, initiating uptake into a phagosome (Weiss & Schaible, 2015). Uptake can be enhanced through the process of opsonisation. Host antibodies and complement components bind to bacterial cells facilitating interactions with Fcγ receptors expressed on phagocytes. Phagosomes undergo a series of fusion and fission events with endocytic organelles to acquire necessary proteins for maturation and subsequent bacterial digestion (Lee *et al*., 2020). Following separation of the phagosome from the cell membrane, early phagosomes are formed and interact with early endosomes. Phagosomes mature over 30 min becoming more acidic, generating a degradative environment (Weiss & Schaible, 2015; Lee *et al*., 2020). Late phagosomes fuse with lysosomes forming the phagolysosome which contains a range of enzymes capable of breaking down proteins, nucleic acids, carbohydrates, and lipids. Most notably, the phagolysosome contains NADPH oxidase, responsible for the generation of ROS and cathepsins (Szulc-Dąbrowska *et al*., 2020).

Neutrophils have been well-documented as the main cell type involved in phagocytosis and intracellular killing of bacterial pathogens. Infection of the mammary gland, extent of disease and resistance to neutrophil phagocytosis and killing differs between *S. uberis* strains. Previous studies reported the *S. uberis* strain C197C to be resistant and EF20 susceptible to bovine neutrophil phagocytosis (Leigh *et* al., 1990; Leigh & Field 1991). Grant & Finch, 1997, investigated the ability for BMMOs to phagocytose and kill these two *S. uberis* strains. Electron micrographs found both strains present within the macrophage phagolysosome, with no significant difference in macrophage bactericidal capacity.

The bactericidal interactions between macrophages and *S. uberis* are complex and appear to depend on the bacterial strain and the source of cells used. BMMOs from lactating cows failed to kill the virulent *S. uberis* strain 0140J; the bacteria increased 2-fold in number within the macrophages 2h post infection. However, macrophages from the mammary gland secretion obtained during the mid-dry period from the same cattle killed 50-65% of phagocytosed *S. uberis* strain 0140J. Additionally, bovine blood derived monocytes were found to exert

significant bactericidal activity against *S. uberis* strain 0140J, suggesting BMMOs were less capable of phagocytosis and the intracellular killing of *S. uberis* (Denis *et al*., 2006). Evaluation of other *S. uberis* strains found that monocyte-derived macrophages were able to kill the nonvirulent strain FSL Z1-124 but failed to kill the virulent strain FSL Z1-048 (Tassi *et al*., 2015).

Encapsulated and non-encapsulated *S. uberis* strains were also compared to investigate resistance to phagocytosis and intracellular killing by BMMOs. Encapsulated strains were found to be more resistant to phagocytosis (43-51% phagocytosed) with reduced bactericidal capacity (25-40%) compared to non-encapsulated strains which were less resistant to phagocytosis (66-74% phagocytosed) with a higher percentage of bacterial cells killed within the BMMO (65-75%) (Almeida & Oliver, 1995). These data suggest that BMMO phagocytosis and intracellular killing of *S. uberis* varies between strains. However, it is yet to be determined the mechanisms *S. uberis* employs to resist BMMO phagocytosis and bactericidal activity.

Previous studies have shown that SUB1154 is required for colonisation and virulence in *S. uberis* strain 0140J (Leigh *et al*., 2010; Archer *et al*., 2020). *S. uberis* evades recognition through TLR2 extracellular expression on bMECs (Günther *et al.,* 2016a) and BMMOs (Chapter 3). Due to the role of SUB1154 in colonisation and immune evasion, it would be beneficial to determine whether SUB1154 delays intracellular killing of *S. uberis* once internalised into BMMOs, prolonging *S. uberis* intracellular survival.

4.1.2. SUB1154 protein between S. uberis strains

S. uberis initiates the immune response in BMMOs through stimulating the production of the pro-inflammatory cytokine IL-1β (Chapter 2). This production of IL-1β from BMMOs challenged with *S. uberis* has been found to be dependent on the expression of the *S. uberis* serine protease SUB1154 (Chapter 3). SUB1154 is covalently anchored to the cell wall by srtA (SUB0881) and shares homology with the *S. pyogenes* ScpA protein. ScpA cleaves the C5a complement factor, inhibiting the recruitment and activation of phagocytic cells to the site of infection (Ward *et al*., 2009; Egan *et al*., 2012). Although no such function has been shown for SUB1154, the protein is required for colonisation and appears to prime the BMMO NLRP3 inflammasome by interacting with TIR domains within the TLR2 signalling pathway (Chapter 3).

ScpA is under control of the *mga* regulon in *S. pyogenes*. Mga is similar to the positive transcriptional regulator, Vru (SUB0144) in *S. uberis* (McIver *et al*., 1995; Ward *et al*., 2009), however SUB1154 appears not to be under transcriptional control of SUB0144 in *S. uberis* (Egan *et al*., 2012). SUB0144 does control the expression of SUB0145 (lactoferrin binding

protein) and SUB1095 (collagen-like surface anchored protein). Like SUB1154, both SUB0145 and SUB1095 are anchored to the *S. uberis* cell surface by srtA (Egan *et al*., 2012). Mutants lacking SUB0145, SUB1095 and SUB1154 were attenuated and failed to induce clinical mastitis, suggesting their important role in colonisation and the pathogenesis of mastitis as their expression was required for full virulence (Leigh *et al*., 2010). However, it has yet to be determined whether SUB0145 and SUB1095 affect the production of IL-1β from BMMOs.

My data has investigated SUB1154 in the context of the *S. uberis* strain 0140J. To fully understand the impact of SUB1154 in *S. uberis* pathogenesis, the expression of SUB1154 and whether SUB1154 directly affects IL-1β production from BMMOs needs to be determined by comparing different *S. uberis* strains.

4.2. Methods

4.2.1. Bacterial culturing conditions

S. uberis strains EF20 (avirulent), 0140J, 0140J mutants (0140JΔ*sub1154*, 0140J::ISS1::*sub1154*, 0140J::ISS1::*0144*, 0140J::ISS1::*0145*, 0140J::ISS1::*sub0881*, 0140J::ISS1::*1095*), C6344, 6780, S6261, C9359, Ab71, C5072, SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were cultured following the protocol outlined in 2.2.3. Mutant strains were generated as previously described by allelic exchange mutagenesis (Leigh *et al*., 2010; Egan *et al*., 2012).

4.2.2. S. uberis mutant strain confirmation

DNA from *S. uberis* strains EF20, 0140J and 0140J mutants were extracted and underwent PCR following the same protocols outlined in 2.2.4 and 2.2.5. Primers (Sigma) used are described below in Table 4.1 (10 µM in DNase free water) using the thermocycling protocol in Table 4.2 for SUB0881, SUB1095 and SUB0144; Table 4.3 for SUB0145 and Table 4.4 for SUB1154. Gel electrophoresis was conducted on the PCR products as described previously (2.2.6) and strain confirmation shown in Fig SI3.

Table 4.1. DNA PCR primers.

Gene target*	Sequence	Function	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)
S. uberis sub0881	CAR41958.1	Sortase A anchoring protein	TGGTTGAAGCAGAAGCTGAA	GGCCACATGATGGATAGCAT	63	949
S. uberis sub1095	CAR42417.1	Collagen like surface anchored protein	CTTTAGCCTCCGCAAGTACAAAT	CTTTAGGTTTCATGTCTGAAGCATT	63	1236
S. uberis sub0144	CAR40575.1	Positive transcription regulator	CATCGAAGGGGGTCTCATTTA	TTGAGAAAGCTGTCCCTGCT	63	692
S. uberis sub0145	CAR40577.1	Lactoferrin binding protein	GGAAACAGGAAATGATCTCAATCT	TCTTCCCAGAGTTGCCTATTTATC	62	1870
S. uberis sub1154	CAR42550.1	Serine protease	CATGAAATGATGATGAGAAATTGAG	GAAATATTTGATCATCCAAAACACC	60	3753

*Gene according to the genomic sequence of *S. uberis* strain 0140J (Ward *et al*., 2009).

Table 4.2. DNA PCR thermocycling protocol for SUB0881, SUB1095 and SUB0144.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	63°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min	1

Table 4.3. DNA PCR thermocycling protocol for SUB0145.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	62°C	30 sec	
Extension	72°C	60 sec	
Final extension	72°C	10 min	1

Table 4.4. DNA PCR thermocycling protocol for SUB1154.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	60°C	30 sec	
Extension	72°C	120 sec	
Final extension	72°C	10 min	1

4.2.3. BMMO challenge

Following isolation, BMMOs were challenged with various stimuli as described in the associated narrative text (section 4.3) and IL-1β measured after 20h. The stimuli used were: heat-killed *S. uberis* strains (outlined in 4.2.1) at an MOI of 50:1 bacterium:BMMO; LPS (10 ng/mL; Millipore, LPS25); rSUB1154 (0.002 μM); inflammasome primer, Pam3CSK4 (1.0 μg/mL; Torcis, 4633) and/or inflammasome activator, silica (500 μg/mL; Sigma, 421553).

4.2.4. Calculating bacterial survival

Bacterial suspensions of *S. uberis* strains 0140J and 0140J*Δsub1154* were washed in PBS and resuspended in IMDM without FBS or antimicrobials at 1 OD. BMMOs were challenged with live *S. uberis* strains 0140J and 0140JΔ*sub1154* at an MOI of 1:1 bacterium:BMMO for 0, 30, 60 and 120 min. Supernatants were collected for determining extracellular bacterial numbers. For intracellular bacterial numbers, BMMOs were washed with PBS + 2% antibiotic-antimycotic three times and then lysed with PBS + 0.5% Tween-20 for 5 min. Samples were serial diluted 10-fold in saline. Cultures were grown on Todd-Hewitt Broth + 1.5% agar plates overnight at 37°C. CFU/mL was calculated by multiplying the average counts by the dilution factor and dividing by the volume added.

4.2.5. Bovine IL-1β ELISA

Detection of bovine IL-1β ELISA was achieved using the Invitrogen Reagent Kit (ESS0027) following the manufacturer's instructions, as previously described (section 2.2.8).

4.2.6. SUB1154 ELISA

SUB1154 concentration was determined for different *S. uberis* strains. Bacterial cultures were resuspended in 1 mL BupH Carbonate/Bicarbonate Buffer (0.2 M, Invitrogen, 28382) and 100 μL added to 96-well plates prior to incubation overnight at 4°C. Plates were aspirated, washed with PBS + 0.05% Tween-20 (Sigma, P1379) and incubated for 30 min at room temperature with blocking buffer (4% BSA and 5% sucrose (Sigma, S0389) in PBS). Wells were washed and incubated for 2h at room temperature with anti-SUB1154 antibody (Egan *et al.*, 2012) diluted in reagent diluent (4% BSA in PBS) at 1:5000. Washing was repeated followed by incubation for a further 2h with secondary antibody (goat anti-rabbit IgG Fc horseradish peroxidase (HRP) conjugated antibody, EMD Millipore, AP156P) diluted in reagent diluent at 1:400. Wells were washed, incubated in the dark for 30 min with substrate buffer (Thermo scientific, N301) and the reaction was stopped using stop buffer (Thermo Scientific, N600).

4.2.7. Western blotting

Bacterial cultures of *S. uberis* strains 0140J, SUD69, SUD276, SUD277 and SUD514 were centrifuged at 5000xg for 3 min and the supernatants were collected and put aside. The pellets were washed three times in PBS by centrifugation at 5000xg for 3 min. Equal volumes of sample (bacterial pellet suspension or supernatant) and loading buffer were heated at 90°C for 5 min and SDS-PAGE performed (as described in 3.2.2).

PVDF membranes (0.2 µm, Biorad, 1704272) were soaked in ethanol and then in 1X semi-dry blot transfer buffer (AlfaAesar, 10X, J63664) along with western blotting stacks (Trans Blot Turbo RTA Transfer kit, Biorad, 1704272). SDS-PAGE gels were blotted onto PVDF membranes using a BioRad Trans Blot Turbo Transfer System for 7 min at 2.5 A. Membranes were blocked overnight with agitation at 4°C in 1X TBS with Tween® (TBST; Thermo Scientific, 20X, J77500-K2) + 2.5g milk powder. Membranes were washed three times for 5 min in TBST and then incubated for 3.5h in TBST + 1% milk powder + 1:5000 anti-SUB1154 antibody. Washing in TBST was repeated, followed by membrane incubation in TBST + 1% milk powder + 1:10,000 anti-rabbit HRP conjugated antibody for 1h. Membranes were washed in TBST 3 times for 5 min, SuperSignal™ Wet Pico Plus Chemiluminescence Substrate (Thermo Scientific, 34580) added for 5 min and images were captured using a Biorad ChemiDoc™ Imaging System.

4.2.8. Phylogenetic analysis

The sequences for the *S. uberis* strains SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were supplied by Dr Daniel Whiley and were aligned to the *S. uberis* strain 0140J sequence on the NCBI database (AM946015.1) using MAFFT software v7.515 (Katoh & Standley, 2013) through command line on Ubuntu. Alignments were then run through the IQ-TREE software v2.2.0.3 (Hoang *et al*., 2018) and the resulting trees were labelled and created using iTOL v6 (Letunic & Bork, 2021).

4.2.9. Target gene sequence analysis between S. uberis strains

Using the NCBI database, the sequences for selected target genes (*sub1154*, *sub0144*, *sub0145*, *sub0881* and *sub1095*) were determined for *S. uberis* strains SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 using *S. uberis* strain 0140J as a reference. Gene sequences were aligned and the amino acid sequence determined using MEGA 11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al*., 2021).

SIFT (Sorting Intolerant From Tolerant) software (Vaser *et al*., 2016) was used to predict whether the amino acid substitutions in SUB1154 would affect protein function. AlphaFold protein structure database (Jumper *et al*., 2021) was then used to predict the SUB1154 3D structure, using The PyMOL Molecular Graphics System, Version 2.5 (Schrödinger LCC, 2023) to visualise and label amino acid positions of interest.

4.2.10. Confocal microscopy

Isolated BMMOs were seeded onto chambered microscopy slides (Labtek 8-well glass slide, 177402). Heat-killed *S. uberis* strain 0140J was incubated in PBS + 20 µM SYT09 (Invitrogen, S4854) for 15 min before challenging BMMOs at an MOI of 1:1 bacterium:BMMO for either 10, 15, 20, 25, 30 or 45 min. Following challenge of BMMOs with *S. uberis* strain 0140J, media was removed and the BMMOs were washed three times by incubation in PBS for 5 min at 37°C to remove remaining extracellular bacteria. BMMOs were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by two washes in PBS. Challenged BMMOs were incubated at room temperature for 20 min with PBS + 0.1% BSA, followed by an overnight incubation of PBS + 0.1% BSA + ElabFluor® 647 anti-mouse F4/80 antibody (5 µL/million cells, F0995M) + 1:1000 DAPI (Invitrogen, D1306). BMMOs were washed three times with PBS for 5 min followed by the removal of all liquid from the chambers. Coverslips (Epredia, 22x50 mm) were mounted using Fluoromount-G (Invitrogen, 00-4958-02) and left to dry overnight at room temperature. Slides were imaged using a Zeiss CellDiscoverer 7 microscope at 50X magnification and images analysed using Fiji software (Schindelin *et al*., 2012).

4.2.11. Statistical analysis

Data was analysed using GraphPad Prism 10.0.3 (GraphPad Software, 2023). Normality was assumed due to the low N value and data was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test. A value of $P \leq 0.05$ was considered to indicate a statistically significant difference. Pearson's correlation coefficient was used to determine association, with nonlinear regression to calculate coefficient of determination.

4.3. Results

4.3.1. Internalisation of S. uberis by BMMOs

Uptake of *S. uberis* strain 0140J by BMMOs was demonstrated using confocal microscopy. *S. uberis* strain 0140J (Fig 4.1A) internalisation into BMMOs was observed 10 min after challenge (Fig 4.1B).

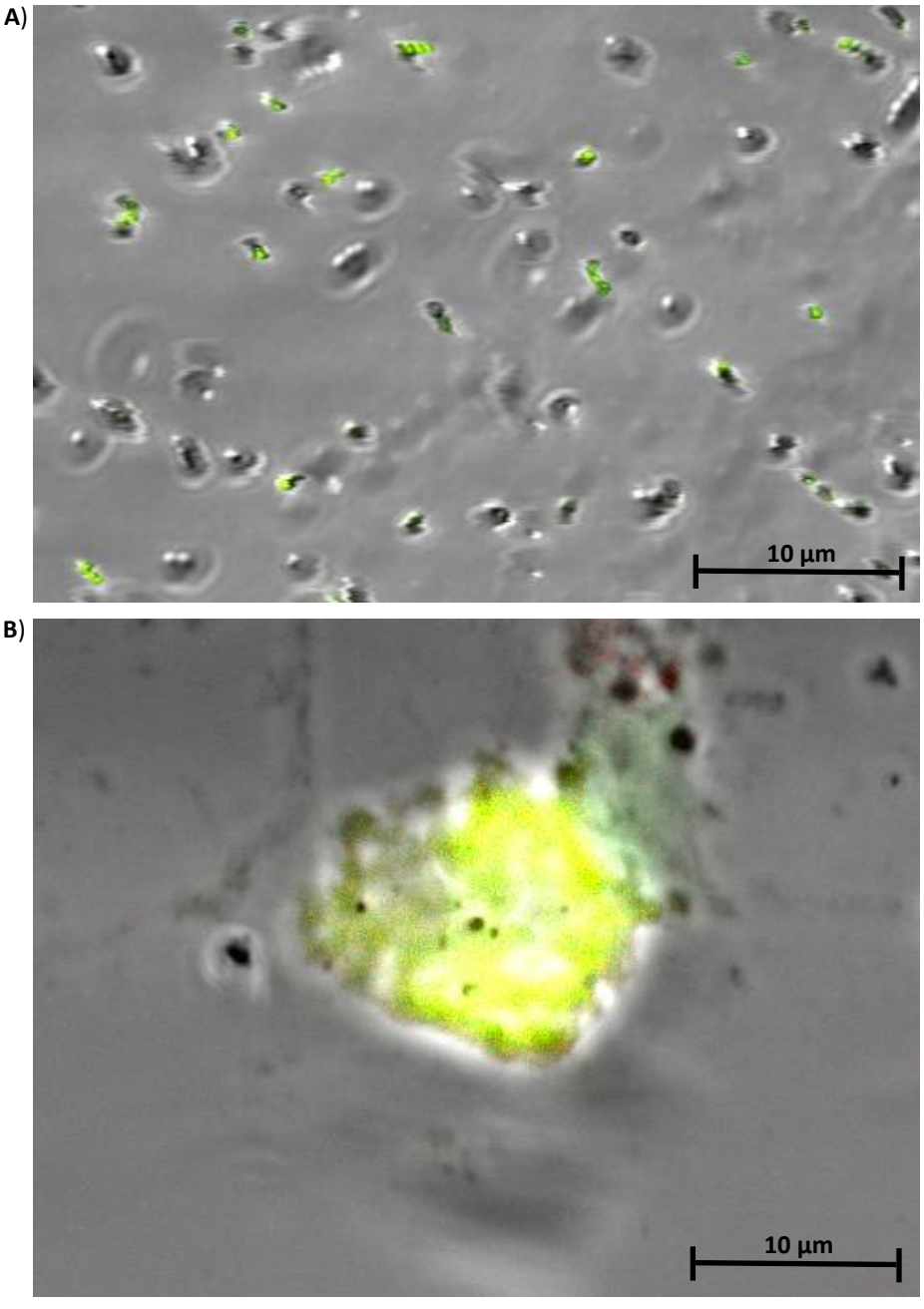


Fig 4.1. Confocal microscopy images of *S. uberis* internalisation by BMMOs. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into a chambered microscopy slide at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J stained with SYT09 at a multiplicity of infection (MOI) of 1:1 *S. uberis* strain 0140J:BMMO for 10 min (**B**). **A**) Stained *S. uberis* strain 0140J only. Following challenge, media was removed, BMMOs washed with PBS and fixed with 4% paraformaldehyde. Coverslips were mounted, slides were imaged using a Zeiss CellDiscoverer 7 microscope at 50X magnification and images were analysed using Fiji software.

4.3.2. Extracellular and intracellular CFU/mL of S. uberis following BMMO challenge

As SUB1154 functions intracellularly, following challenge of BMMOs with $5x10^4$ *S. uberis* strains 0140J (Fig 4.2A) and 0140JΔ*sub1154* (Fig 4.2B), the bacterial cell counts in the extracellular environment and following internalisation into BMMOs were measured over 120 min. The number of *S. uberis* bacteria found in the extracellular environment gradually decreased to minimal values over the time-course, with no difference observed between *S. uberis* strains 0140J and 0140JΔ*sub1154*. Conversely, increasing bacterial cell numbers were found intracellularly, with the greatest number recorded at 120 min. The intracellular number of *S. uberis* strain 0140J bacteria ($8.25x10^4$) was greater than that of *S. uberis* 0140JΔ*sub1154* ($4.75x10^4$) at 120 min (and greater than the initial number of bacteria in the assay).

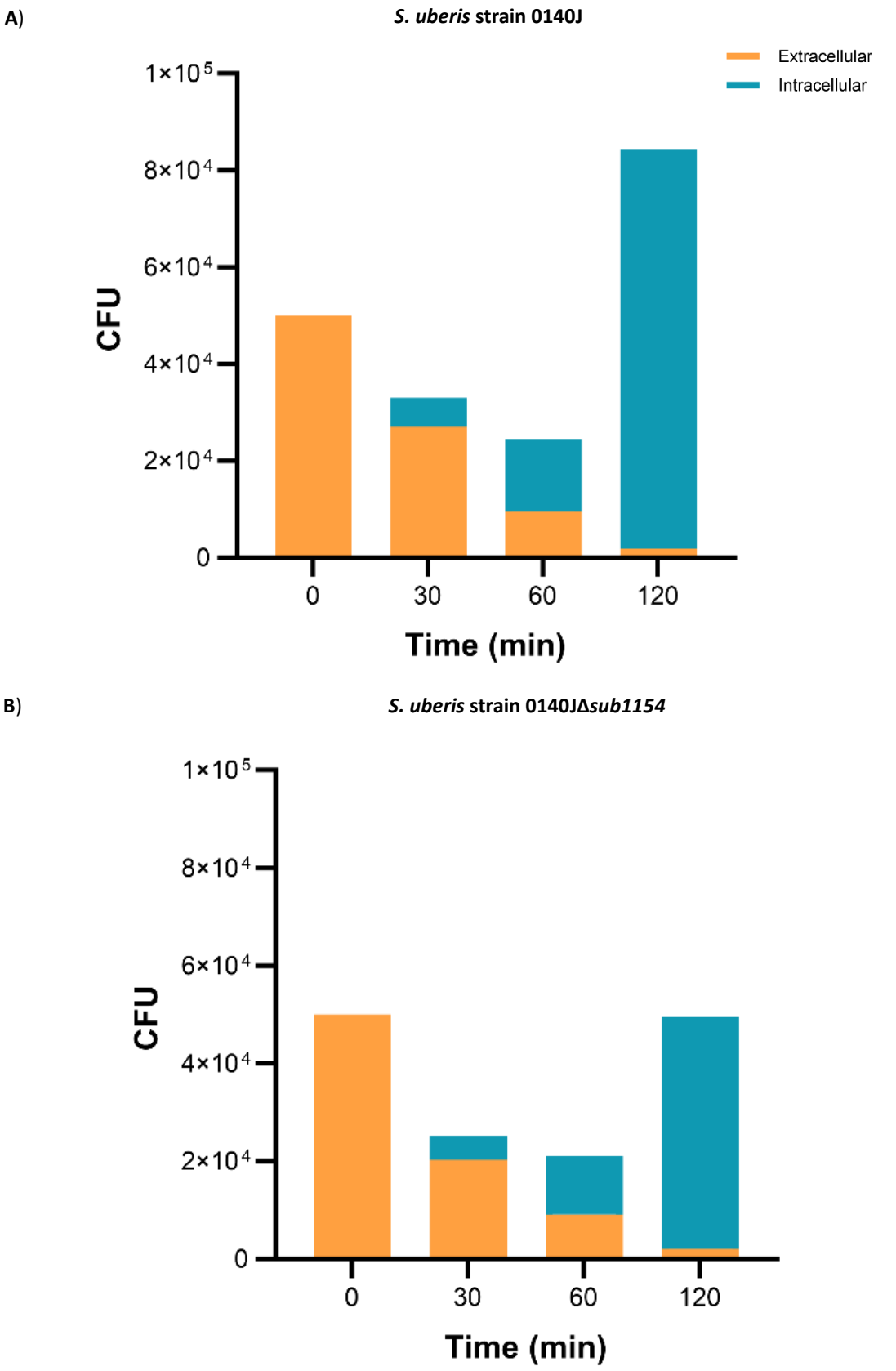


Fig 4.2. Extracellular and intracellular CFU/mL of *S. uberis* following BMMO challenge. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with live *S. uberis* strain 0140J (**A**) or the SUB1154 deletion mutant (0140JΔ*sub1154*) (**B**) at a multiplicity of infection (MOI) of 1:1 bacterium:BMMO for 120 min. Supernatants were removed to determine extracellular bacterial numbers. BMMOs were washed and subsequently lysed to determine intracellular bacterial numbers. CFU/mL was calculated by growing dilutions on Todd-Hewitt agar plates overnight at 37°C.

4.3.3. Differences in the IL-1β response between S. uberis strain 0140J virulence gene mutants

Previous studies investigated several *S. uberis* virulence genes through the generation of isogenic mutants carrying an inactivating ISS1 insertion and challenge of the bovine mammary gland (Leigh *et al*., 2010; Egan *et al*., 2012). BMMOs were challenged with these mutants and the production of IL-1β was measured (Fig 4.3). Stimulation of BMMOs with all of the mutants resulted in a significant reduction in IL-1β production ($P<0.0001$) compared to *S. uberis* strain 0140J, with strain 0140J::ISS1:*sub0144*, 0140J::ISS1:*sub1154* and 0140JΔ*sub1154* showing the greatest IL-1β reduction.

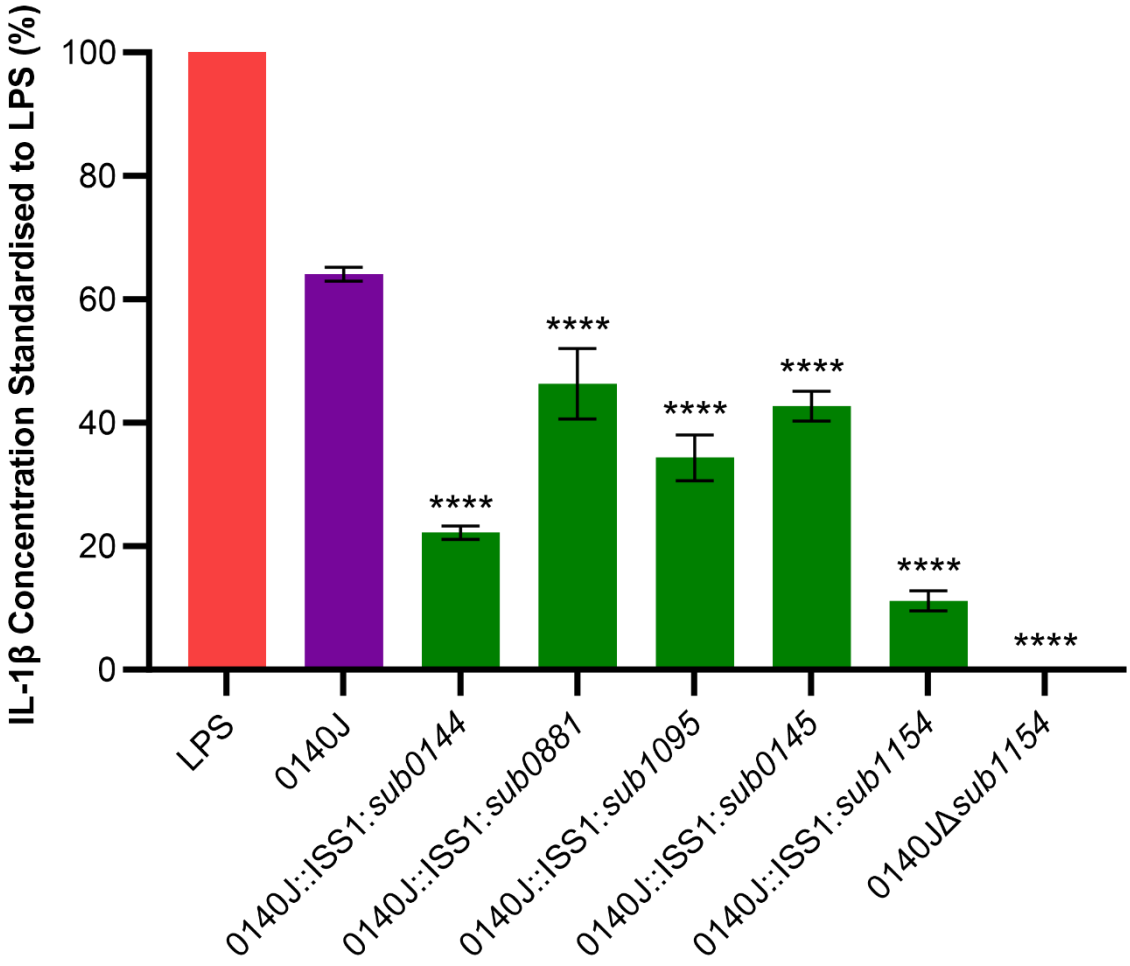


Fig 4.3. BMMO IL-1β production following stimulation with *S. uberis* strain 0140J virulence gene mutants. Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strains 0140J or 0140J isogenic mutants carrying an inactivating ISS1 insertion: 0140J::ISS1:*sub0144*, 0140J::ISS1:*sub0881*, 0140J::ISS1:*sub1095*, 0140J::ISS1:*sub0145*, 0140J::ISS1:*sub1154* or 0140J SUB1154 deletion mutant (0140JΔ*sub1154*) at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO. Supernatants were collected after 20h and the concentration of IL-1β was measured by ELISA after 20h. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (****$P<0.0001$ compared to *S. uberis* strain 0140J).

4.3.4. Differences in SUB1154 expression and the IL-1β response between S. uberis strains isolated from clinical mastitis

BMMOs were also challenged with different *S. uberis* strains isolated from clinical cases of mastitis (Hossain *et al*., 2015) as well as the avirulent strain EF20. The IL-1β produced by BMMOs following stimulation (Fig 4.4A) and the concentration of SUB1154 expressed on these strains (Fig 4.4B) were measured. All strains were found to either express significantly more (EF20, S6261, C9359) or less (C6344, 6780, Ab71, C5072) SUB1154 compared to *S. uberis* strain 0140J ($P<0.0001$). However, these strains produced significantly less IL-1β following BMMO stimulation compared to *S. uberis* strain 0140J ($P<0.0001$ for C6344, 6780, C9359 and Ab71; $P<0.05$ for EF20), except strains S6261 and C5072 where no difference was found.

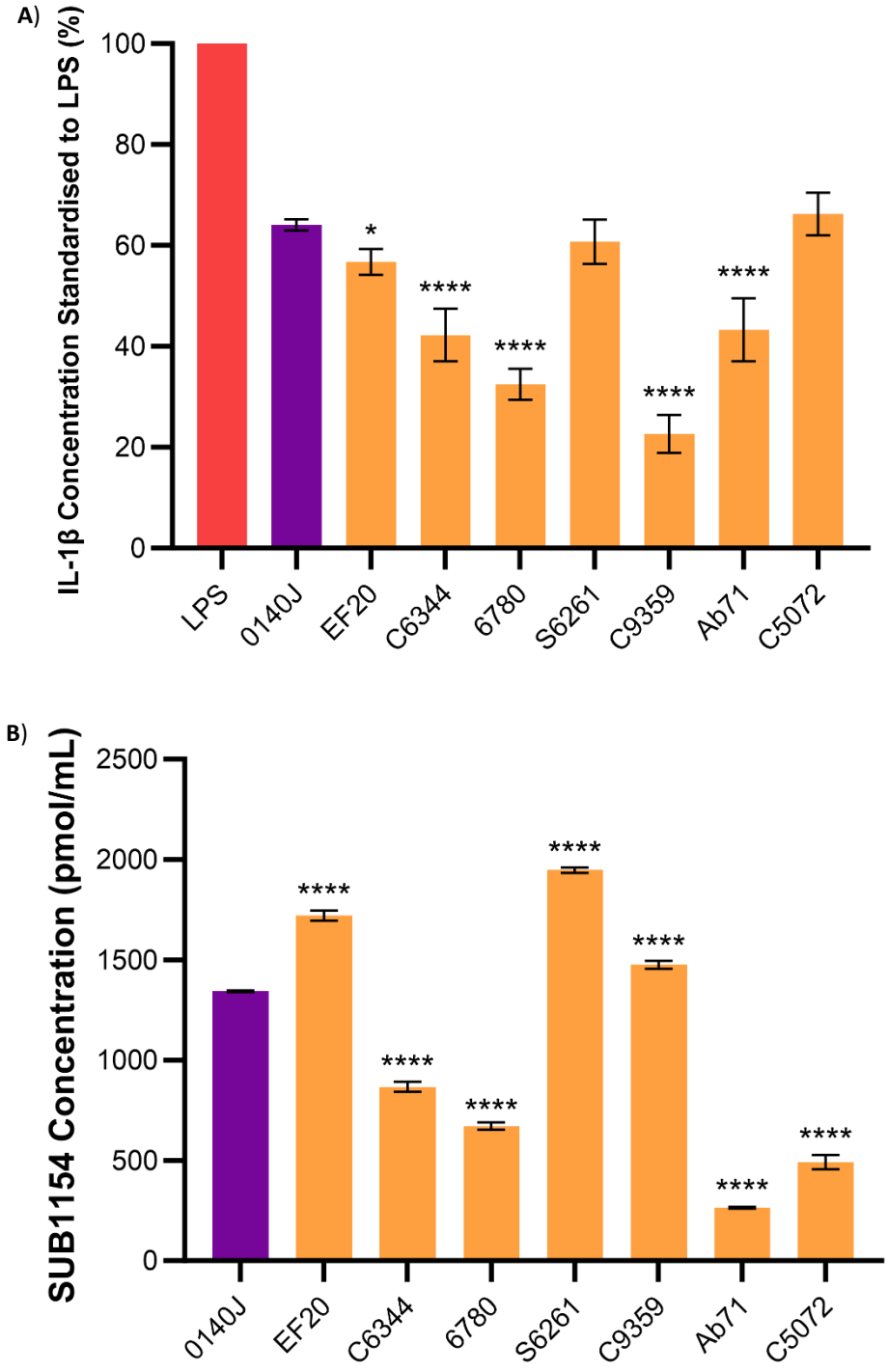


Fig 4.4. SUB1154 expression on *S. uberis* strains isolated from clinical mastitis and IL-1β production from BMMOs following challenge. A) Bovine mammary macrophages (BMMOs) were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strain 0140J or strains isolated from clinical mastitis: EF20 (avirulent), C6344, 6780, S6261, C9359, Ab71 and C5072 at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO. Supernatants were collected after 20h and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=6±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*****P*<0.0001; **P*<0.05 compared to *S. uberis* strain 0140J). **B**) *S. uberis* strains 0140J, EF20, C6344, 6780, S6261, C9359, Ab71 and C5072 were cultured and concentration of SUB1154 determined by ELISA. Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*****P*<0.0001 compared to *S. uberis* strain 0140J).

4.3.5. Differences in SUB1154 expression and the IL-1β response between S. uberis strains

Additionally, BMMOs were challenged with *S. uberis* strains isolated from the bovine mammary gland (disease status unknown). The IL-1β produced by BMMOs following stimulation (Fig 4.5A) and the concentration of SUB1154 expressed on these strains (Fig 4.5B) were measured. IL-1β produced from BMMOs challenged with these strains varied in comparison to *S. uberis* strain 0140J stimulation. Most notably, there was minimal IL-1β production from BMMOs challenged with strains SUD69 and SUD514. The SUD69 and SUD514 strains also displayed minimal SUB1154 expression.

SUD276 and SUD277 strains also expressed low concentrations of SUB1154. However, BMMOs challenged with SUD276 produced no difference in IL-1β and SUD277 stimulation caused significantly more IL-1β production compared to *S. uberis* strain 0140J ($P<0.01$). Strains SUD76 and SUD221 expressed no significant difference in SUB1154 compared to *S. uberis* strain 0140J. BMMOs challenged with SUD76 produced significantly less IL-1β ($P<0.01$), whereas SUD221 stimulation produced significantly more IL-1β ($P<0.05$) compared to *S. uberis* strain 0140J. BMMOs challenged with strains SUD285 and SUD511 produced the greatest concentration of IL-1β. SUD285 also expressed the highest concentration of SUB1154, however, SUD511 expressed approximately half the level of SUB1154 compared to SUD285. SUD248, SUD249 and SUD250 expressed similar concentrations of SUB1154 but only SUD249 resulted in increased production of IL-1β from BMMOs ($P<0.001$) compared to *S. uberis* strain 0140J.

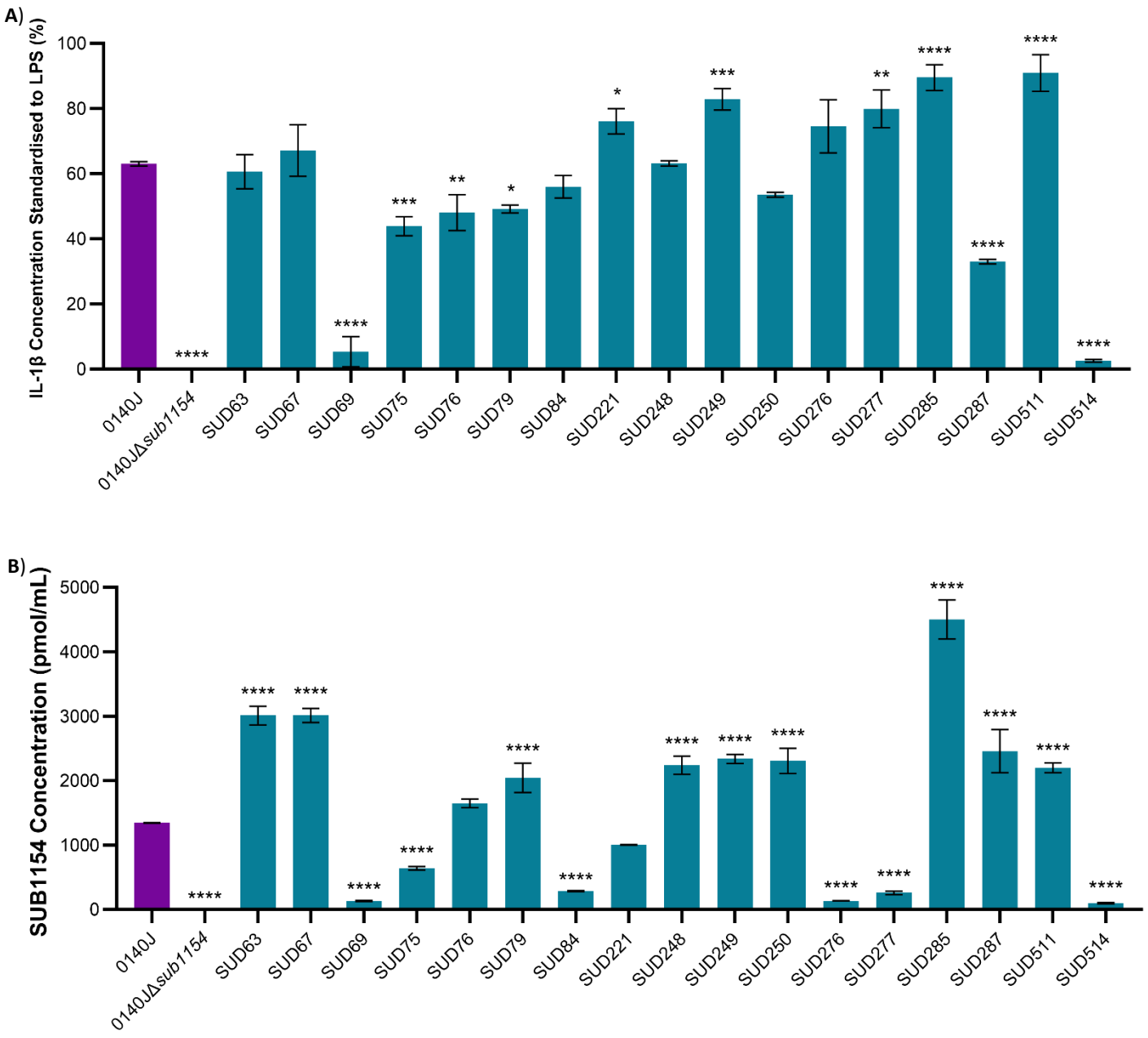


Fig 4.5. Differential expression of SUB1154 between *S. uberis* strains and IL-1β production following BMMO challenge. A) BMMOs were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strains 0140J, 0140J SUB1154 deletion mutant (0140JΔ*sub1154*) or strains isolated from the bovine mammary gland: SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO; **C**) 0.002 µM rSUB1154 protein; Pam3CSK4 (1.0 µg/mL; primes the inflammasome) and/or silica (500 µg/mL; activates the inflammasome). Supernatants were collected after 20h and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*****P*<0.0001; ****P*<0.001; ***P*<0.01 or **P*<0.05 compared to *S. uberis* strain 0140J). **B**) *S. uberis* strains 0140J, 0140JΔ*sub1154,* SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were cultured and concentration of SUB1154 determined by ELISA. Data is presented as N=3±SD (normality assumed) and was statistically analysed using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*****P*<0.0001 compared to *S. uberis* strain 0140J).

A moderate positive correlation was found between the concentration of SUB1154 expressed on *S. uberis* strains and the production of IL-1β from stimulated BMMOs (r = 0.49) with a weak coefficient of determination (R^2 = 0.24) (Fig 4.6).

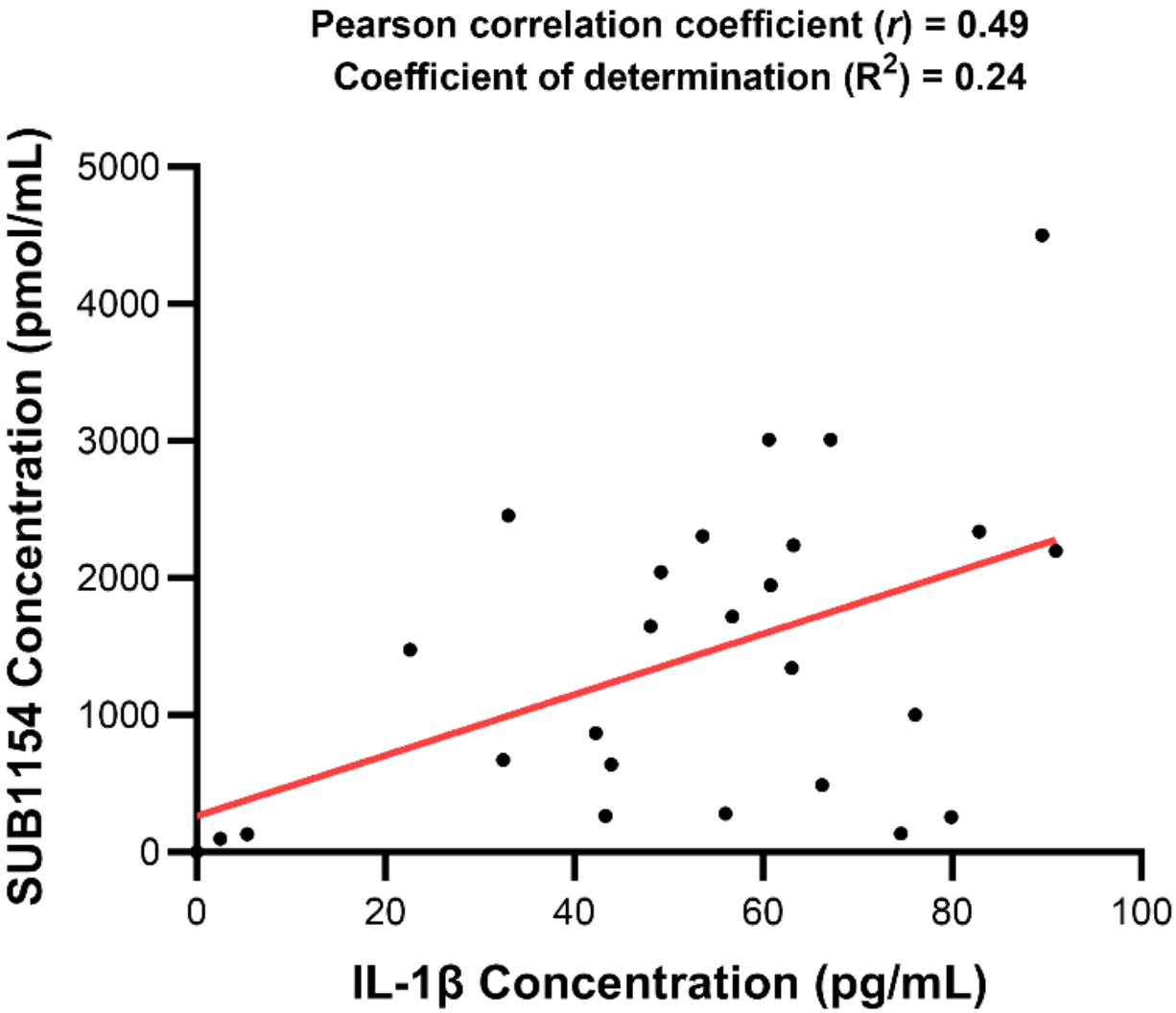


Fig 4.6. Correlation between concentration of SUB1154 expressed on *S. uberis* strains and the production of IL-1β from stimulated BMMOs. Association between the SUB1154 expression and the IL-1β concentration produced from BMMOs stimulated with *S. uberis* strains isolated from the bovine mammary gland were determined using Pearson correlation coefficient (positive correlation, r = 0.49). Nonlinear regression calculated the line of best fit and the coefficient of determination (R^2 = 0.24).

Restoration of IL-1β production from BMMOs was found following challenge with SUD69 or SUB514 and rSUB1154 protein (P<0.0001 and P<0.001 respectively) (Fig 4.7). There was no difference in IL-1β production from BMMOs following stimulation with SUD69 or SUD514 and the inflammasomal activator silica. An increase in IL-1β was observed when BMMOs challenged with SUD69 or SUD514 and the inflammasome primer Pam3CSK4.

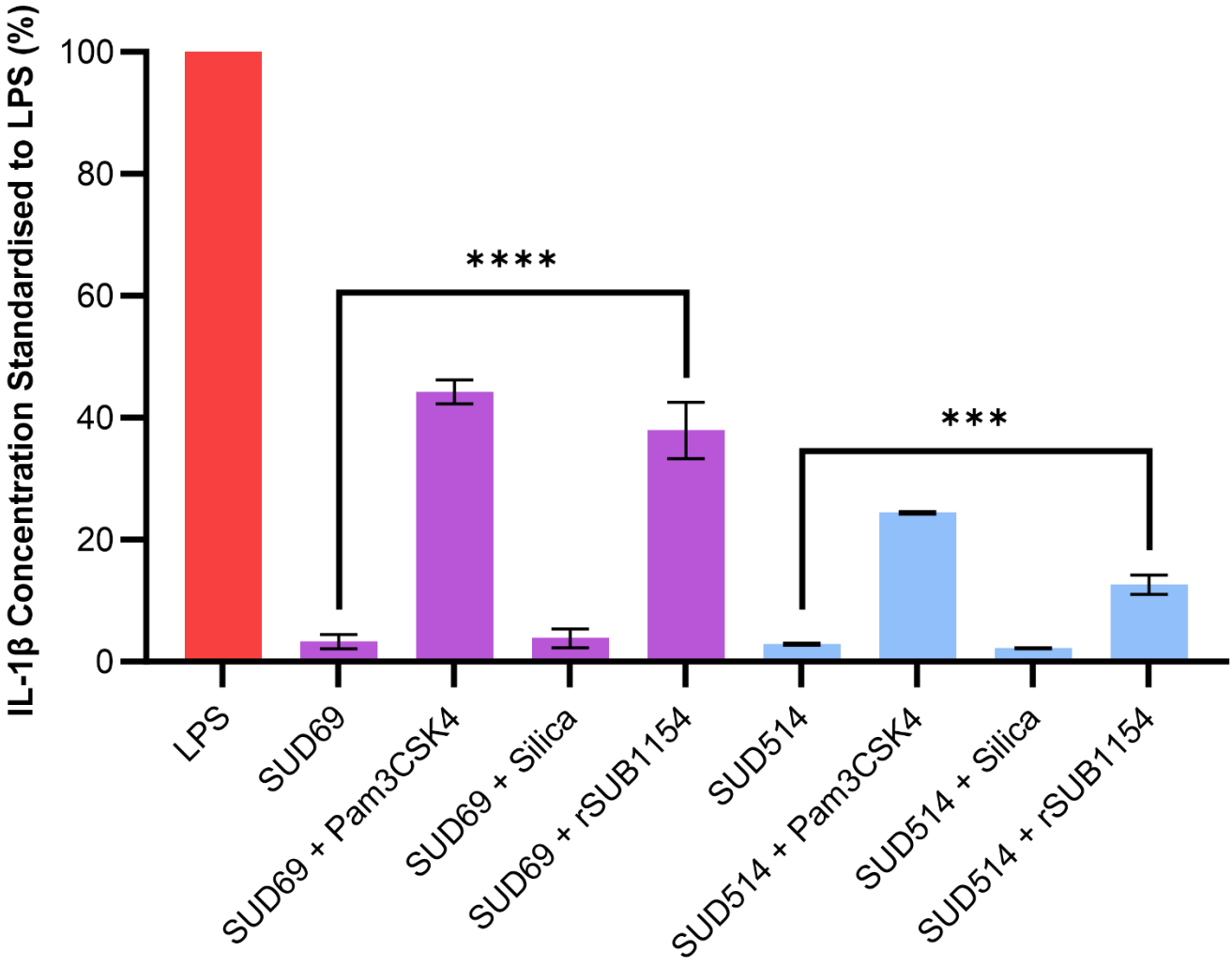


Fig 4.7. IL-1β production from BMMOs challenged with *S. uberis* strains SUD69 and SUD514 in the presence of rSUB1154. BMMOs were isolated from milk and seeded into culture dishes at ~50,000 BMMOs/well. BMMOs were challenged with heat-killed *S. uberis* strains isolated from the bovine mammary gland: SUD69 and SUD514 at a multiplicity of infection (MOI) of 50:1 bacterium:BMMO; 0.002 µM rSUB1154 protein; Pam3CSK4 (1.0 µg/mL; primes the inflammasome) and/or silica (500 µg/mL; activates the inflammasome). Supernatants were collected after 20h and the concentration of IL-1β was measured by ELISA. BMMOs were also unstimulated in a no treatment group and this mean was deducted from the other values, which were then standardised to the LPS positive control (10 ng/mL). Data is presented as N=3±SD (normality assumed) and was statistically analysed using a Tukey multiple comparisons post hoc test (****P<0.0001; ***P<0.001).

The detection of SUB1154 protein within the pellet and supernatant of *S. uberis* strains 0140J, SUD69, SUD276, SUD277 and SUD514 was measured using western blotting (Fig 4.8). SUB1154 was more greatly detected in the pellet of *S. uberis* strain 0140J compared to the supernatant. Strains SUD69 and SUD276 had a comparatively lower level of SUB1154 within the pellet, with a greater concentration observed in the supernatant. High detection levels of SUB1154 were found in both the pellet and supernatant of SUD277, whereas SUB1154 was barely detected in the pellet of SUD514 but was found at a greater concentration in the supernatant. Evidence of SUB1154 cleavage was observed in the supernatants of all the *S. uberis* strains, with some evidence of cleavage in the pellet of SUD277.

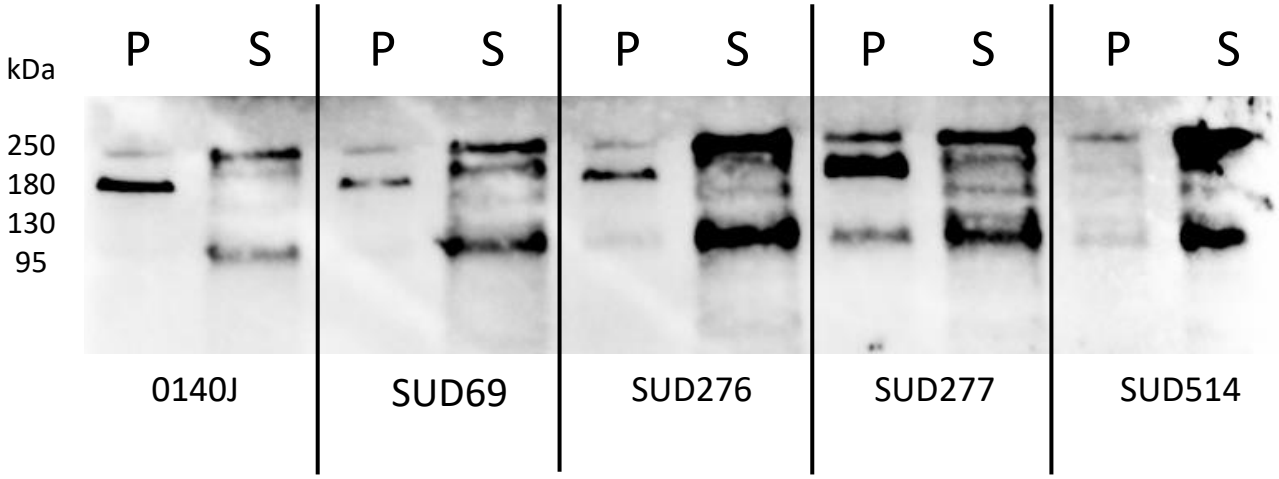


Fig 4.8. Detection of SUB1154 in the pellet and supernatant of *S. uberis* strains. *S. uberis* strains 0140J, SUD69, SUD276, SUD277 and SUD514 were cultured overnight and centrifuged. The supernatants were collected, and the pellets washed. Samples were heated, run on the same SDS-PAGE gel and western blotting performed (lines added to aid interpretation). P = pellet, S = supernatant.

4.3.6. Phylogenetic tree of different S. uberis strains

The production of IL-1β from BMMOs varied following stimulation with the different *S. uberis* strains isolated from the bovine mammary gland. To investigate whether the differences in IL-1β production correlated with the *S. uberis* strains genotypes, a phylogenetic tree was produced in order to group the *S. uberis* strains by genetic distance (Fig 4.9). No relationship was found between IL-1β production from stimulated BMMOs (Fig 4.5A) and the genetic distances or grouping of the *S. uberis* strains.

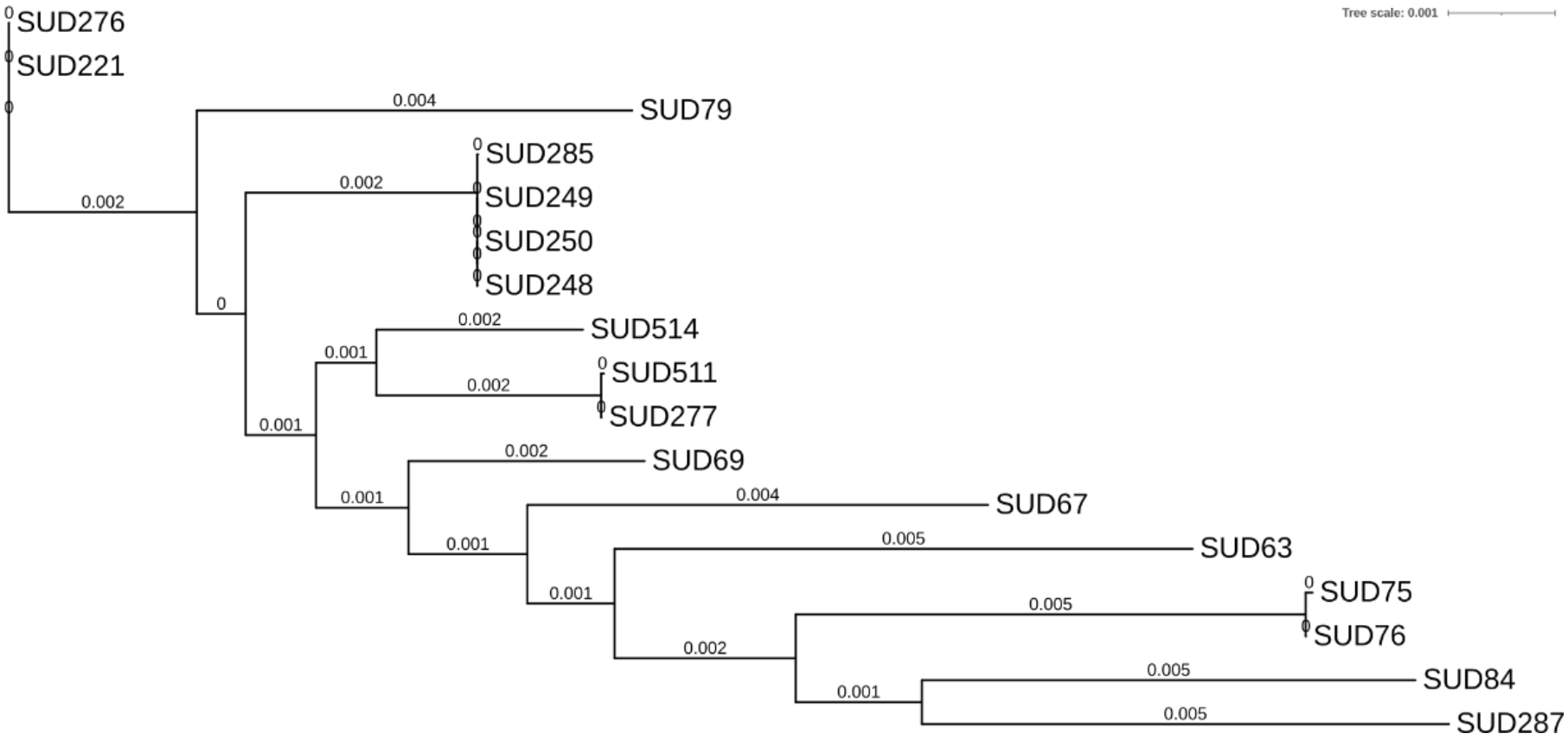


Fig 4.9. Phylogenetic tree of *S. uberis* strains. *S. uberis* strains isolated from the bovine mammary gland: SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were sequenced and aligned using command line software MAFFT to the *S. uberis* strain 0140J. The phylogenetic tree was generated using IQ-TREE and the figure created using iTOL. Branch length is shown above each line.

4.3.7. Predicting whether amino acid changes in the SUB1154 protein affects protein function

Further investigations were completed to determine whether the differences in IL-1β production from BMMOs correlated to changes in the amino acid sequence of specific gene targets between *S. uberis* strains. Analysis of the amino acid sequences of *sub0144, sub0145, sub0881, sub1095* and *sub1154* were compared between the *S. uberis* strains isolated from the bovine mammary gland and *S. uberis* strain 0140J. No mutations of significant interest that explain the differences in IL-1β production from BMMOs or SUB1154 expression were identified (Table SI2). Amino acid changes in the SUB1154 protein between the *S. uberis* strains were further analysed.

SIFT software was used to predict whether the amino acid changes in the SUB1154 protein would affect protein function, resulting in the changes in SUB1154 expression and/or IL-1β production from stimulated BMMOs between the *S. uberis* strains. SIFT selected 16 sequences that were similar to SUB1154 (Table 4.5) to determine this.

Table 4.5. SIFT selected sequences similar to SUB1154.

Protein target	Host species	Function	Homology to SUB1154 (%)
ScpA	*S. pyogenes* (Q8K5Q0)	C5a peptidase	32.1
ScpA	*S. pyogenes* (Q8NZ80)	C5a peptidase	32.1
ScpA	*S. pyogenes* (P15926)	C5a peptidase	31.9
ScpA	*S. pyogenes* (P58099)	C5a peptidase	31.9
ScpA	*S. pyogenes* (Q5X9R0)	C5a peptidase	31.7
P1P	*Lactococcus lactis cremoris*	(Lactocepin) breaks down milk proteins during growth.	11.6
P2P	*Lactococcus lactis cremoris*	(Lactocepin) breaks down milk proteins during growth.	11.7
P3P	*Lactococcus lactis cremoris*	(Lactocepin) breaks down milk proteins during growth.	11.2
P2P	*Lacticaseibacillus paracasei*	(Lactocepin) breaks down milk proteins during growth.	11.4
Vpr	*Bacillus subtilis*	Minor extracellular serine protease.	11.2
Pyrolysin	*Pyrococcus furiosus*	Endopeptidase activity towards caseins.	10.4
Cucumisin	*Cucumis melo*	Allergen with autocatalytic cleavage.	9.9
TTP2	*Caenorhabditis elegans*	Component of the proteolytic cascade. Role in regulation of fat storage.	8.2
TTP2	*Schizosaccharomyces pombe*	Component of the proteolytic cascade. Role in regulation of fat storage.	7.9
Subtilisin	*Bacillus licheniformis*	Extracellular alkaline serine protease catalyses the hydrolysis of proteins.	7.5
CWBP23	*Bacillus subtilis*	Cell-wall associated serine protease involved in peptide bridges.	6.5

P1P, PI type wall associated serine protease; P2P, PII type wall associated serine protease; P3P, PIII type wall associated serine protease; TTP2, tripeptidyl peptidase 2.

The amino acid substitutions were scored from 0 to 1. A score of >0.05 indicated tolerance, with <0.05 predicting altered protein function that would be considered damaging. SIFT predicted 18 substitution positions to be tolerated and 22 that could affect protein function (Table 4.6). Of the amino acid changes predicted to affect protein function, 8 were found to be within the median sequence conservation range (2.75-3.50).

Table 4.6. SUB1154 amino acid substitutions predicted to be tolerated or affect protein function.

Substitution position	From	To	Affect or tolerate function	SIFT Score	Median sequence conservation	Sequences represented at this position
1	M	I	Affect	0.00	4.32	1
7	H	N	Affect	0.00	4.32	1
78	S	P	Affect	0.03	3.36	11
79	K	N	Tolerated	0.12	3.36	11
80	S	N	Tolerated	0.05	3.36	11
96	L	T	Affect	0.03	3.36	11
118	K	N	Tolerated	1.00	3.36	11
157	L	I	Tolerated	0.33	3.40	10
167	E	Q	Tolerated	0.15	3.40	10
216	S	N	Tolerated	0.92	3.36	14
219	G	S	Tolerated	0.45	3.35	15
229	I	V	Tolerated	0.15	3.35	15
291	A	S	Tolerated	0.26	3.40	10
336	A	V	Tolerated	0.23	3.35	12
379	V	F	Affect	0.04	3.36	11
474	A	S	Tolerated	0.22	3.35	13
558	A	G	Tolerated	0.23	3.35	13
561	A	G	Affect	0.01	3.35	13
612	L	I	Tolerated	0.31	3.40	10
632	R	S	Tolerated	1.00	3.44	6
641	N	K	Affect	0.03	3.40	10
664	P	S	Tolerated	0.26	3.44	6
755	G	V	Affect	0.04	3.44	6
822	R	K	Tolerated	1.00	3.44	6
955	T	I	Affect	0.00	3.44	6
1012	N	S	Tolerated	0.14	3.44	6
1018	H	S	Affect	0.00	4.32	1
1030	S	Y	Affect	0.03	3.43	2
1037	H	Q	Tolerated	0.34	3.43	2
1056	G	D	Affect	0.00	4.32	1
1077	S	F	Affect	0.00	4.32	1
1080	S	R	Affect	0.00	4.32	1
1084	K	E	Affect	0.00	4.32	1
1090	A	T	Affect	0.00	4.32	1
1100	Y	H	Affect	0.00	4.32	1
1100	Y	R	Affect	0.00	4.32	1
1109	S	C	Affect	0.00	4.32	1
1122	F	L	Affect	0.00	4.32	1
1126	A	T	Affect	0.00	4.32	1

SUB1154 sequences in the *S. uberis* strains isolated from the bovine mammary gland: SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were compared to the SUB1154 sequence of *S. uberis* strain 0140J to identify amino changes. SIFT software was used to predict whether these changes in amino acids would be tolerated or affect protein function by comparing to 16 other sequences similar to SUB1154 (Table 4.5). SIFT score of >0.05 was regarded as tolerated (light blue) with <0.05 affecting (dark blue) protein function. Median sequence conservation was used to measure the diversity of the sequences used for prediction, with the optimal range between 2.75 and 3.50. Sequences represented at this position shows the number of sequences that SIFT identified as similar to base predictions from having an amino acid at that position.

Evaluation of SIFT analysis identified three amino acid substitution positions that were most likely to affect SUB1154 protein function (Table 4.7). *S. uberis* strains SUD69, SUD79, SUD248, SUD248, SDU250, SUD285 and SUD514 all had the same amino acid at positions 78, 641 and 955 as the *S. uberis* strain 0140J. However, only SUD69 and SUD514 expressed less SUB1154, with the other strains all expressing a greater concentration of SUB1154 compared to the mean. Amino acid substitution at position 641 from asparagine (N) to lysine (K) only occurred in conjunction with the substitution at position 78. A reduction in IL-1β production was found when lysine was present at position 641, however, this did not correlate with low SUB1154 expression. In comparison, an increase in IL-1β production was found when serine was substituted to proline at position 78. Similarly, this did not correlate with increased SUB1154 expression. *S. uberis* strains with threonine (T) substituted for isoleucine (I) at position 955 resulted in increased IL-1β production from BMMOs, with only SUD277 expressing lower concentrations of SUB1154. No obvious trend was associated with the change in amino acids at any of the three sites of interest and the expression of SUB1154 protein or production of IL-1β from BMMOs stimulated with the different *S. uberis* strains.

Table 4.7. Comparison of amino acid changes at three positions in SUB1154 between different *S. uberis* strains.

	Amino acid position				
***S. uberis* strain**	**78**	**641**	**955**	**IL-1β (pg/mL)**	**SUB1154 (pmol/mL)**
0140J	S	N	T	63.02	1344.12
SUD63	S	N	I	60.60	3009.47
SUD67	P	N	T	67.11	3010.81
SUD69	S	N	T	5.33	131.37
SUD75	P	K	T	43.89	638.37
SUD76	P	K	T	48.05	1647.40
SUD79	S	N	T	49.18	2045.14
SUD84	P	K	I	56.00	283.18
SUD221	P	N	T	76.10	1003.79
SUD248	S	N	T	63.18	2238.82
SUD249	S	N	T	82.82	2337.72
SUD250	S	N	T	53.56	2305.55
SUD276	P	N	T	74.54	133.34
SUD277	S	N	I	79.90	257.79
SUD285	S	N	T	89.53	4501.73
SUD287	P	K	T	33.03	2457.19
SUD511	S	N	I	90.95	2198.93
SUD514	S	N	T	2.52	96.613
Mean				**57.74**	**1646.74**

SUB1154 sequences in the *S. uberis* strains isolated from the bovine mammary gland: SUD63, SUD67, SUD69, SUD75, SUD76, SUD79, SUD84, SUD221, SUD248, SUD249, SUD250, SUD276, SUD277, SUD285, SUD287, SUD511 and SUD514 were compared to the SUB1154 sequence of *S. uberis* strain 0140J to identify amino changes. SIFT analysis resulted in three amino acid substitution positions that are most likely to affect protein function. S = serine, P = proline, N = asparagine, K = lysine, T = threonine, I = isoleucine. Mean IL-1β was calculated from the concentration of IL-1β produced from BMMOs 20h after challenge with the *S. uberis* strains (Fig 4.5A). Mean SUB1154 was calculated from the concentration of SUB1154 protein expressed on the *S. uberis* strains (Fig 4.5B). Red indicates IL-1β or SUB1154 concentrations lower than the mean with green indicating concentrations greater than the mean.

The locations within the SUB1154 protein in which these three amino acid positions are situated may contribute to their effect on protein function. The SUB1154 3D structure was predicted using AlphaFold (Fig 4.10A) and substitution positions 78, 651 and 955 were visualised and labelled on the domains (Fig 4.10B) and 3D structure (Fig 4.10C) of the SUB1154 protein using PyMOL. Substitution position 78 was found to be located in a loop with low confidence. Position 641 and 955 were found to be situated with high confidence within an anti-parallel β-barrel and a β-sheet respectively.

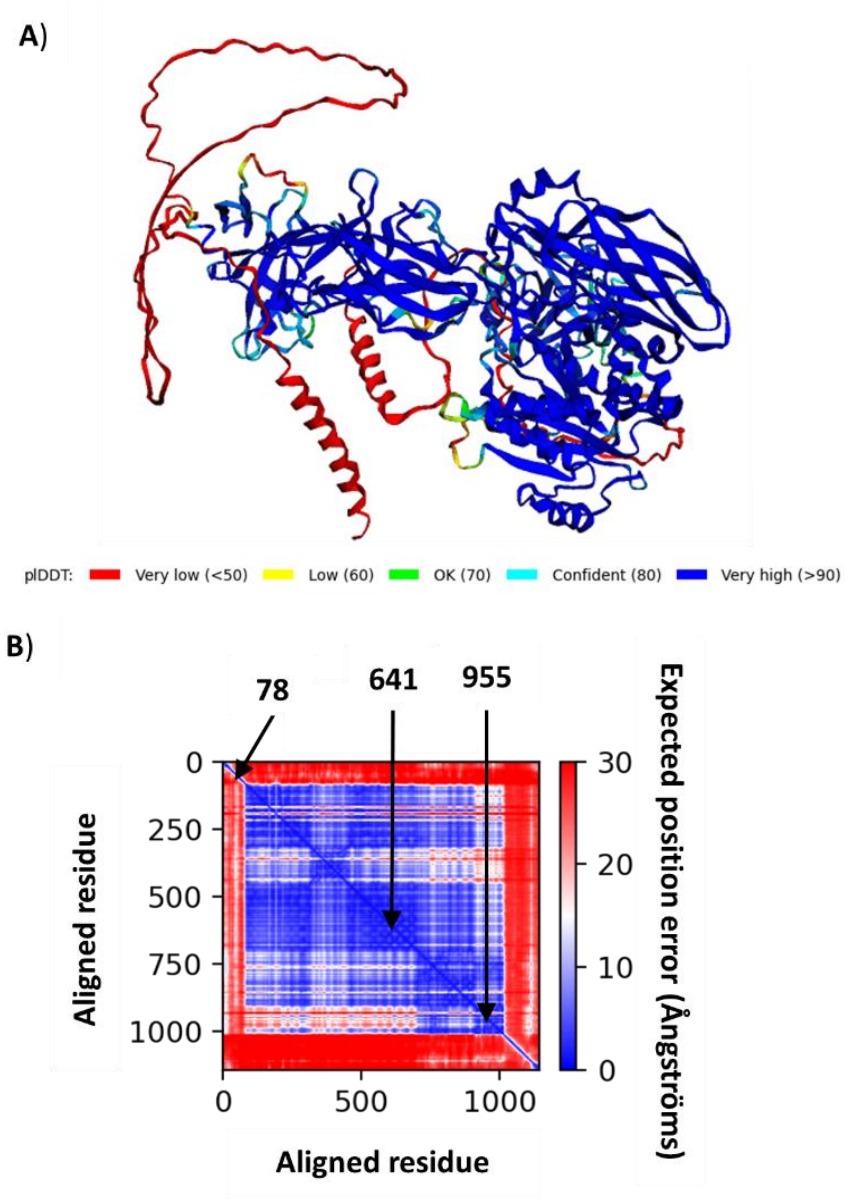


Fig 4.10. Predicted SUB1154 protein structure and the positions of the three substitutions of interest. AlphaFold was used to predict the structure of the SUB1154 protein and PyMOL was used to visualise and label the images created. **A**) Predicted structure containing atomic coordinates and per-residues confidence estimates on a scale from 0 to 100, with higher scores corresponding to higher confidence (plDDT). **B**) Predicted aligned error allows for splitting of the protein into reliably understood domains which show a higher confidence in their structure. **C**) Positions 78, 641 and 955 (SIFT predicted mutations at these sites affect protein function) were labelled onto the 3D SUB1154 predicted structure. Together the images show that position 78 is located in a low confidence area of prediction, with positions 641 and 955 within β-sheets that display high levels of prediction confidence.

4.4. Discussion

S. uberis initiates the immune response in BMMOs through stimulating the production of the pro-inflammatory cytokine IL-1β. This production of IL-1β has been found to be dependent on the expression of the *S. uberis* serine protease SUB1154. To fully understand the impact of SUB1154 in *S. uberis* pathogenesis, the expression of SUB1154 and whether SUB1154 directly affects IL-1β production from BMMOs needs to be determined by comparing different *S. uberis* strains.

4.4.1. Effect of SUB1154 on S. uberis internalisation into BMMOs

Evidence of *S. uberis* internalisation into BMMOs was found 10 min after challenge. *In vivo*, internalisation by BMMOs may be delayed due to the dilution of *S. uberis* within the milk and the presence of milk proteins that BMMOs also uptake. The total number of *S. uberis* cells decreased from challenge with $5x10^4$ cells to $2.5x10^4$ cells 60 min after challenge. This decrease in *S. uberis* cell numbers could be due to the digestion of *S. uberis* by BMMOs. Denis *et al*., 2006, found that 2h post infection, macrophages only killed 50-65% of phagocytosed *S. uberis*. Therefore, this accounts for the initial decrease in *S. uberis* cell numbers. After 120 min, *S. uberis* bacteria have had time to replicate and in combination with the inefficient killing by BMMOs. This could explain the increase in *S. uberis* cell numbers at 120 min.

Involvement of SUB1154 in *S. uberis* intracellular survival was determined by challenging BMMOs with either *S. uberis* strain 0140J or the SUB1154 deletion mutant (0140JΔ*sub1154*). The proportion of extracellular and intracellular *S. uberis* cells remained the same in the presence and absence of SUB1154 up to 60 min after challenge. However, by 120 min there was $3x10^4$ more intracellular *S. uberis* strain 0104J compared to *S. uberis* 0140JΔ*sub1154*. Previous studies have shown that colonisation is reduced in *S. uberis* strains lacking SUB1154 (Leigh *et al*., 2010); further supporting the role of SUB1154 in intracellular growth of *S. uberis*.

4.4.2. The relationship between the SUB1154 protein and IL-1β production from BMMOs

Different *S. uberis* strains isolated from the bovine mammary gland induced BMMOs to produce different concentrations of IL-1β. Production of IL-1β is SUB1154-dependent and a moderate positive correlation, with a weak coefficient of determination, was found between the concentration of SUB1154 expressed on *S. uberis* strains and the production of IL-1β from stimulated BMMOs. However, it was discovered to be more complex than simply greater SUB1154 expression causing greater IL-1β production.

S. uberis strains SUD84, SUD276 and SUD277 all expressed low concentrations of SUB1154 but induced production of IL-1β from BMMOs at a similar level as *S. uberis* strain 0140J, raising causality into question. This suggests that the extent of IL-1β produced is not proportional to the expression of SUB1154. Therefore, if it is not the NLRP3 inflammasome priming signal (SUB1154) affecting IL-1β production it could be due to the activation signal. To add to this, BMMOs challenged with the isogenic mutant of SUB0144 resulted in decreased IL-1β production compared to the genetically intact parental strain 0140J, despite SUB0144 not controlling the expression of SUB1154 (Egan *et al*., 2012). On the other hand, SUB0144 does regulate the expression of SUB0145 and SUB1095, which could contribute to the NLRP3 inflammasome activation signal. Although the function of SUB1095 is unknown, SUB0145 was suggestive to have many functions, one of which was to facilitate *S. uberis* internalisation into bMECs (Ward *et al*., 2009). It could be speculated that SUB0145 may also facilitate internalisation into BMMOs, with more *S. uberis* cells intracellularly available to provide the NLRP3 inflammasome activation signal. Consequently, this would also increase intracellular SUB1154 available for NLRP3 inflammasome priming. The amino acid sequences for SUB0144 were compared between *S. uberis* strains, however, no substitutions of significant interest were found (Table SI3). Further investigation is therefore required to determine the NLRP3 inflammasome activation signal(s) of *S. uberis* and whether these correlate with the extent of IL-1β production from BMMOs following challenge.

S. uberis strains SUD69 and SUD514 initially followed the correlation with low SUB1154 expression and low IL-1β production from BMMOs. When recombinant SUB1154 was introduced following BMMO challenge with SUD69 or SUD514, there was an increase in IL-1β production; indicating the low level of inflammasome priming. However, genome sequence data indicated both strains contained SUB1154 in a form and context that would not be expected to impact expression (Table SI2; Table SI4). An issue with the SUB1154 ELISA protocol was that the centrifugation and wash steps meant only SUB1154 proteins expressed on the bacterial cells were measured. Therefore, SUB1154 western blotting was performed, detecting SUB1154 in the bacterial pellet and the culture suspension supernatant. This revealed that there was minimal SUB1154 expressed on the bacterial cells within the pellet, coinciding with the ELISA data, but there was evidence of SUB1154 in the supernatant. Therefore, the low SUB1154 expression and subsequent low IL-1β production in context of SUB69 and SUB514 was due to the removal of the protein.

SrtA (SUB0881) covalently anchors SUB1154 to the *S. uberis* cell wall (Egan *et al*., 2012). The amino acid sequence of SUB0881 was compared between the *S. uberis* strains to determine

whether a substitution within SUD69 and SUD514 resulted in the absence of SUB1154 expressed on the bacterial cells. However, no substitutions of significant interest were evident (Table SI4). There is potential for other unknown proteins which affect SUB1154 anchoring or control the release of SUB1154 from the cell wall. Alternatively, there may be variation in stability of the cell wall proteins between *S. uberis* strains that affect SUB1154 anchoring and release. Further genome analysis is required to try and identify such proteins related to SUB1154. BMMOs challenged with the insertional mutants of SUB0881 or SUB1154 (lacks the sortase anchoring domain, Archer *et al*., 2020) resulted in decreased IL-1β production from BMMOs compared to the genetically intact parental strain 0140J. However, IL-1β production was not ablated. Following internalisation of such a strain, the protein and bacterial cell do not necessarily get co-transported inside the BMMO, consequently reducing the SUB1154 available for intracellular interactions that result in inflammasome priming.

4.4.3. The effect of amino acid changes on SUB1154 protein function

Initial analysis found no substitutions of significant interest in the SUB1154 amino acid sequence between the *S. uberis* strains. Further analysis using SIFT software was conducted to determine whether any of the amino acid substitutions observed (Table SI2) had the potential to affect the function of SUB1154. SIFT analysis selected 16 reference sequences that were similar to the *S. uberis* strain 0140J SUB1154 protein. The SUB1154 sequence of each of the *S. uberis* strains were compared to the reference sequences to predict which substitutions were likely to alter SUB1154 function. Understanding the functions of the reference proteins could provide insight into additional activities of SUB1154.

4.4.3.1. SIFT reference proteins

The homology of the reference protein sequences to SUB1154 ranged from 6.5 to 32.1%. *S. pyogenes* ScpA sequence displayed the highest homology, followed by Lactocepin which is located on the cell envelope of *Lactococcus lactis cremoris*. Lactocepin hydrolyses casein (80% of the protein found in milk) into smaller amino acids and peptides, allowing bacteria to utilise these molecules during growth. Lactocepin also exerts anti-inflammatory effects. For example, lactocepin in *Lactobacillus* selectively degrades the chemokine CXCL10. The Group A streptococcal protease ScpC is a member of the lactocepin family with a similar structure and selectively degrades the pro-inflammatory cytokine IL-8 (Visser *et al*., 1991; Schillde *et al*., 2012). SUB1154 primes the NLRP3 inflammasome contributing to the bovine pro-inflammatory response. Therefore, it would be interesting to determine whether SUB1154 could also act in an anti-inflammatory manner by also degrading CXCL10 and IL-18.

Another reference protein identified by SIFT was the extracellular subtilase pyrolysin produced by the marine hyperthermophilic archaeon *Pyrococcus furiosus*. Ca^{2+} ions were shown to both destabilise and stabilise the pyrolysin enzyme, with destabilisation promoting enzyme activity by lowering the activation energy (Zeng *et al*., 2014). Pyrolysin was also demonstrated to have endopeptidase activity as all caseins were degraded following incubation with the enzyme (Voorhorst *et* al., 1996). Although caseins are not present in the natural environment of *P. furiosus*, the ability for pyrolysin to degrade caseins and be destabilised/stabilised by Ca^{2+} ions in conjunction with 10% homology to SUB1154, further suggests SUB1154 could play a role in casein degradation in milk to promote *S. uberis* colonisation.

Similar functions have also been reported for the other reference proteins. The serine proteases subtilisin (*Bacillus licheniformis*), Vpr and CWBP23 (*Bacillus subtilis*) catalyse the hydrolysis of proteins and peptide amines. Enzyme activity of these serine proteases is also enhanced by the presence of Ca^{2+} ions (Stephenson & Harwood, 1998; Foophow *et al*., 2022). Tripeptidyl-peptidase 2 (TTP2) is a multifunctional and evolutionarily conserved protease, most notably involved in fat storage and MHCI antigen processing (McKay *et al*., 2007; Endert, 2008).

Despite low homology of these reference sequences to SUB1154, their functions could indicate potential roles for SUB1154 that could be investigated. For example, Ca^{2+} ions may enhance activity of SUB1154 to digest casein, promoting bacterial growth. Therefore, the reduced colonisation observed in the *S. uberis* strain 0140JΔ*sub1154* could be due to lack of access to amino acids and peptides. Therefore, despite the pro-inflammatory actions of SUB1154, the protein is essential for colonisation and so persists within the genome. SIFT identified that SUB1154 is a protease based on the comparison proteins used, however, IL-1β production is not dependent on the protease activity of SUB1154. Comparison of SUB1154 to other proteins that prime the NLRP3 inflammasome at an intracellular location may enable SIFT to be a more accurate predictor of functional sequence variation in this instance.

4.4.3.2. Three substitution positions that might affect SUB1154 protein function

SIFT analysis detected 22 amino acid changes that were predicted to affect SUB1154 function. Further evaluation condensed the list to the substitution positions that were most probable to affect SUB1154 function. The first exclusion criteria assessed the number of reference sequences. Substitution positions were only included if the SUB1154 comparison was against >5 reference sequences as the more sequences SUB1154 were compared to, the higher the chance the amino acid change will affect SUB1154 function.

The biochemical properties of the remaining 7 amino acid substitutions were then assessed. Substitution positions 379, 651 and 755 were excluded as the amino acid change did not alter the polarity or charge (all remained non-polar and neutral). Amino acid substitution at position 96 was only observed in *S. uberis* strain SUD84 and so was also excluded. This resulted in three substitution positions of interest: 78, 641 and 955.

At position 78, *S. uberis* strains either expressed amino acid serine (polar, neutral) or proline (non-polar, neutral). Non-polar (hydrophobic) amino acids are important in protein folding kinetics and therefore substitution of serine to proline may affect protein structure, hence altering protein function (Huang *et al*., 2012). *S. uberis* strains that possessed only the proline amino acid substitution all caused increased production of IL-1β from BMMOs following challenge. Potentially, this substitution may alter SUB1154 folding, promoting higher affinity binding to TIR domains. However, position 78 is situated within a loop of low confidence on the predicted SUB1154 protein structure. Therefore, it is unlikely that this amino acid substitution at this position would affect SUB1154 function.

Amino acid substitution at position 641 was always found in conjunction with the substitution at position 78. In combination, *S. uberis* strains resulted in decreased production of IL-1β from stimulated BMMOs. At position 641, *S. uberis* strains either expressed asparagine (polar, neutral) or lysine (polar, positive). Position 641 is located, in high confidence, near a β-hairpin within a β-barrel, where one side of the β-sheet faces the core and the other points towards the environment. Positively charged amino acids, such as lysine, contribute to protein stability by binding to negatively charged amino acids within the core or on neighbouring β-strands. Lysine is one of the most favourable amino acids to be paired with aspartate or glutamate within β-hairpins. The amino acid change to lysine in *S. uberis* strains at position 641 could increase protein stability, reducing affinity to TIR domains leading to reduced IL-1β production from BMMOs (Riemen & Waters, 2009; Kim *et al*., 2016).

Similar to substitution position 78, *S. uberis* strains either expressed polar neutral threonine or non-polar neutral isoleucine at position 955, suggesting altered protein folding. In contrast to position 78, position 955 is situated within a high confidence β-sheet with a SIFT score of 0. This indicates that an amino acid substitution at this position confidently alters protein function. *S. uberis* strains expressing isoleucine at position 955 all caused increased IL-1β production from stimulated BMMOs. This may suggest an increased affinity of SUB1154 to TIR domains due to altered protein folding but this requires additional detailed investigation.

Conclusion

SUB1154 appears to play a positive role in *S. uberis* intracellular survival/proliferation within BMMOs. This may contribute towards the reduced virulence of the SUB1154 deletion mutant. This aspect requires further investigation to confirm and extend these findings. BMMOs challenged with different *S. uberis* strains induced varying concentrations of IL-1β. However, no evidence was found to support the greater/lesser production of IL-1β being related to sequence variation within SUB1154 or the amount of SUB1154 expressed by the *S. uberis* strains. The extent of IL-1β production could be speculated to be dependent on the NLRP3 inflammasome activation signal(s), which requires further elucidation. SIFT software and subsequent analysis identified three substitution positions that are predicted to affect SUB1154 function. Substitution to isoleucine at position 955 was the most likely to affect SUB1154 function. This is because of the low SIFT score, localisation within a high confidence β-sheet and the affect non-polar amino acids have on protein folding, in conjunction with *S. uberis* strains expressing isoleucine causing an increased IL-1β production from BMMOs. This may be due to an increased affinity of SUB1154 to TIR domains. Despite the predictive impact of the three amino acid substitutions affecting SUB1154 function, there is lack of evidence to conclude these mutations are important. Further investigation is required to evaluate binding affinity of SUB1154 to TIR domains between the different *S. uberis* strains.

Chapter 5: Discussion

According to the literature, *S. uberis* differs to most bacterial pathogens as upon entry into the mammary gland it does not interact with bMECs (Günther *et al*., 2016a & 2016b). Instead, the immune response is initiated through BMMOs. Due to the activation of epithelial cells occurring secondarily, this results in the delayed recruitment of neutrophils to the mammary gland, allowing *S. uberis* to colonise (Egan *et al*., 2012). The host immune response against *S. uberis* results in damage to the mammary gland leading to clinical signs of infection. The interaction between *S. uberis* and BMMOs dictates the outcome of infection and understanding more about this host pathogen interaction involved in early pathogenesis may offer opportunities to intervene and reduce disease. This thesis adds new mechanistic information and further expands upon the understanding of how this interaction occurs.

5.1. Importance of utilising BMMOs

A variety of cells have been used experimentally to investigate mastitis pathogenesis including the murine macrophage cell line RAW 246.7, bovine epithelial cell line MAC-T cells and blood derived bovine macrophages. Typically, macrophages are classified as M1 or M2 depending on whether they act in a pro- or anti- inflammatory manner. Recent developments in the field of macrophage biology depict that this is a restrictive method of classification as macrophages are completely different depending on their environmental niche and specific cellular properties. This underpins the importance of using macrophages from the bovine mammary gland, as in this work, as they are likely to generate a different response to macrophages differentiated from blood monocytes. This is evident as TLR2 was concluded to be involved in the recognition of *S. uberis* when conducted in a murine model (Wan *et al*., 2020), which was subsequently disproven in the context of primary bovine cells (Günther *et al.,* 2016a). Some studies have utilised BMMOs, however, there was a lack of a standardised isolation protocol in the literature. Here, a highly reproducible method has been developed for the isolation of BMMOs.

5.2. BMMO immune response to S. uberis

Macrophages recognise invading bacteria through receptor binding or by chance during immune surveillance in which macrophages sample the extracellular environment (Paape *et al*., 2000). *S. uberis* is a Gram-positive bacterium and so canonically would be expected to interact with TLR2 expressed on the macrophage extracellular surface via LTA and/or other

lipopeptides. As previously mentioned, bMECs also express TLR2 and *S. uberis* was found not to interact with these cells via this receptor (Günther *et al.,* 2016a). Results in the present work demonstrate that *S. uberis* also does not bind to TLR2 expressed on the extracellular surface of BMMOs. This was demonstrated by *S. uberis* activation of BMMOs in the presence of MMG 11, which should block binding of extracellular TLR2. To add to this, when cell entry into BMMOs was inhibited so that *S. uberis* could no longer be internalised, the BMMOs remained inactive. Together, this suggests that *S. uberis* avoids the host immune system by not binding to the TLR2 LRR domains and initiation of the inflammatory response only occurs following internalisation into BMMOs during immune surveillance.

In vivo, activated macrophages secrete pro-inflammatory cytokines (TNF-α and IL-1β) and chemokines (IL-8) to stimulate neighbouring epithelial and immune cells. Upon activation, stimulated cells also produce such mediators. Collectively, this results in the recruitment of other immune cells, such as neutrophils and lymphocytes, to the site of infection to aid in bacterial clearance (Denis *et al*., 2006). IL-1β is a major inflammatory mediator produced by macrophages and signifies immune activation of the cells. Therefore, the concentration of IL-1β was measured as an indicator for the BMMO response to *S. uberis.* Previous research outlined several *S. uberis* virulence factors of interest. The *S. uberis* strain 0140J mutant lacking SUB1154 was found to colonise to a lesser extent compared to the genetically intact parental strain 0140J (Leigh *et al*., 2010). Also, there was ablation of IL-1β produced from BMMOs stimulated with the SUB1154 deletion mutant (Archer *et al*., 2020). Subsequently, BMMOs challenged with the deletion mutant and recombinant SUB1154 protein resulted in partial restoration of IL-1β production. This highlighted the importance of the SUB1154 protein in the initiation of the immune response in BMMOs. SUB1154 is a serine protease that is covalently anchored to the *S. uberis* cell wall. Due to its homology to the *S. pyogenes* ScpA protein, it was originally hypothesised to function as a C5a peptidase (Ward *et al*., 2009; Egan *et al*., 2012), however, no evidence has been provided to support this claim and its function remains unknown.

5.3. Localisation of SUB1154 following internalisation by BMMOs

The ability to visualise the localisation of SUB1154 following *S. uberis* internalisation by BMMOs would provide further evidence for its intracellular role. Staining of *S. uberis* was successful following the method described in 4.2.10, however, the F4/80 antibody for staining BMMOs did not work. This may be due to the antibody being targeted against human cells; therefore, a bovine alternative would need to be used, or a different macrophage stain.

Potentially, fluorophore conjugated to CD14 antibody could be used, however, flow cytometry interpretation found that CD14 may not be expressed on all BMMOs or not be completely cross reactive. After establishing a successful method to stain BMMOs, SUB1154 antibody conjugated to a different fluorophore could then also be used to show SUB1154 localisation.

5.4. SUB1154 primes the NLRP3 inflammasome through BMMO TIR domain interactions

IL-1β is produced by macrophages stimulated with *S. uberis* as the end-product of the NLRP3 inflammasomal pathway. NLRP3 inflammasome assembly and activity is controlled by two stages, priming and activation. Normally during the priming stage, bacterial PAMPs bind to TLRs on the extracellular surface and trigger a signalling cascade that results in the activation of NF-κB which translocates into the nucleus and increases transcription of *nlrp3* and *pro-il-1β* (Bauernfeind *et* al., 2009; Franchi *et al*., 2009; Gong *et al*., 2020; Duez & Pourcet, 2021). Post-translational modifications then maintain NLRP3 in an inactive configuration until activation threshold is reached (Yang *et al*., 2017; Duez & Pourcet, 2021). There are a variety of different stimuli that can act as NLRP3 inflammasome activation signals, either from extracellular stimuli or the accumulation of molecules intracellularly. Some examples include the release of cathepsins from the lysosome (Hornung *et al*., 2008), movement of ions (Muñoz-Planillo *et al*., 2013) and the presence of ROS following mitochondrial damage (Mullen *et al*., 1985; Gong *et al*., 2018). Once the inflammasome receives both priming and activating signals, pro-IL-1β is cleaved into its active form by caspase-1 and is secreted out of the BMMO to initiate an immune response (Yang *et al*., 2017; Swanson *et al*., 2019; Duez & Pourcet, 2021).

Transcriptional analysis and utilisation of the NLRP3 inflammasome primer Pam3CSK4 and activator silica, in combination with *S. uberis* 0140JΔ*sub1154* and rSUB1154 challenge of BMMOs concluded that SUB1154 primes the NLRP3 inflammasome. This conflicts with data found in Archer *et al*., 2020, that suggested SUB1154 activates the NLRP3 inflammasome. Further investigation found that caspase-1 activity was unaffected by the presence or absence of rSUB1154, suggesting SUB1154 acts upstream of NF-κB. As *S. uberis* was found to not interact with TLR2 extracellularly, the intracellular TIR domain of TLR2 was inhibited and following BMMO challenge with *S. uberis*, IL-1β production was ablated. This indicates that SUB1154 primes the NLRP3 inflammasome through BMMO TIR domain interactions. Further investigation is required to provide more evidence for this interaction and to acquire the specific mechanism of action.

5.5. Investigating SUB1154:TIR domain interactions

Four proteins in BMMOs contain potential TIR domains that could be involved in the interaction with SUB1154: TIR domains in TLR2, TIRAP, MyD88 or IL-1R proteins. Initially, it is important to determine which of these proteins SUB1154 binds to.

5.5.1. Pull-down assay

This could be achieved by using a pull-down assay in which the anti-SUB1154 antibody is attached to beads in a column. Purified recombinant SUB1154 protein would be run through the column, allowing the protein to bind to the antibody on the beads. BMMOs would then be isolated, lysed and run through the column. BMMO proteins would interact and bind to SUB1154; washing would remove any unbound proteins. Additionally, BMMOs could be challenged with rSUB1154 and then lysed and run through the column. The SUB1154-protien interaction would remain in the column adhered to the beads and then eluted. SDS-PAGE and western blotting analysis could be conducted on the eluted proteins with the membrane probed with antibodies against SUB1154, TLR2, MyD88 and TIRAP. The presence of the BMMO proteins on the membrane would indicate their binding to SUB1154 (Louche *et al*., 2017). A potential issue with using western blotting is the lack of bovine specific antibodies to these relatively uncommon protein targets. To resolve this, specific antibodies could be produced.

Alternatively, the membrane could be probed with the SUB1154 antibody only as this is available and known to be successful in western blot analysis. The BMMO protein bound to SUB1154 could be inferred through the respective band sizes on the membrane for bovine TLR2 (90.2 kDa/3513 bp, UniProt A0A0P0QLR2), MyD88 (33.7 kDa/2578 bp, UniProt Q599T9) and TIRAP (24.4 kDa/785 bp, UniProt Q2LGB6) proteins in addition to the size of the SUB1154 protein. However, protein-protein interactions during cellular processes, such as cell signalling, are normally transient (Phizicky & Fields, 1995; Golemis, 2002). Therefore, this could result in the eluted proteins becoming dissociated from each other. Additionally, the heating stage of the SDS-PAGE would likely disrupt these protein-protein interactions. Therefore, probing the membrane with SUB1154 antibody only would result in the other proteins remaining undetected. The use of mass spectrometry (MS) could be a method to prevent such a false negative result.

5.5.2. Mass spectrometry

Affinity purification MS may be used for protein-protein interaction analysis. This method utilises tagging the protein of interest with compounds, such as Strep-Tag. This reduces the

need for antibodies and the tagged SUB1154 protein would function as an affinity capture probe. The bound proteins would then undergo MS analysis and size would be used to determine which BMMO protein is interacting with SUB1154. Alternatively, crosslinking MS may be used in which protein-protein interactions are covalently linked by chemical crosslinkers, overcoming the issue regarding transient interactions becoming dissociated. This is followed by MS analysis and localisation of the specific cross-linked amino acid residues may be determined using computational modelling via database searching (Richards *et al*., 2021). Previous research found bacterial-host protein-protein interactions using MS. Penn *et al*., 2018, used affinity purification MS to generate a protein-protein interaction map between *Mycobacterium tuberculosis* and human macrophages. This led to the discovery that the host macrophage ubiquitin ligase CBL interacts with and attenuates the *M. tuberculosis* virulence factor, LpqN, which acts as a switch for host anti-bacterial and anti-viral responses. A strep-tactin resin tag was used and specific interactions were determined using the MiST bioinformatic algorithm.

5.5.3. Phage display

Alternatively, phage display could be utilised to determine which Tcp SUB1154 interacts with. A phage library could be created by inserting each of the TIR genes (TLR2, TIRAP and MyD88) into different phage, which would express each of the Tcps of interest on their surface. This library could then be exposed to the rSUB1154 protein. Only the Tcp expressing affinity for SUB1154 would adhere and the unbound phage would be washed away. The bound phage could then be eluted and amplified by *E. coli* infection. This process would be repeated 2-3 times and the amplified phage could be sequenced to determine which Tcp is present (New England Biolabs, 2023).

5.5.4. SUB1154 interactions specifically with the TIR domain of BMMO Tcps

After determining which BMMO Tcp interacts with SUB1154, evidence needs to be provided that this interaction is specifically via the host TIR domain. Bacteria, such as *E. coli*, could be genetically engineered to produce either the bovine TLR2, MyD88 or TIRAP proteins using plasmids. Mutated versions of these bovine proteins could also be created with the TIR domains deleted. The refined protein-protein interaction method could then be repeated. If the Tcp becomes absent when the TIR domain is deleted, this would suggest that SUB1154 interacts specifically with the host TIR domain. Alternatively, a series of non-overlapping Tcp peptides between 9 to 14 amino acids could be derived to identify whether these block the interaction with SUB1154 using ELISA (Ward *et al*., 2008).

Proteins bind to each other through a combination of hydrophobic bonding, van der Waals forces and salt bridges at specific binding domains on each protein. Having determined that it is the TIR domain within the bovine Tcp that binds to SUB1154, the next discovery should be at which part of the SUB1154 protein this interaction is occurring. Generation of peptides could also be utilised here, however, SUB1154 is a very large protein and would require a large number of peptides to be tested. Despite the predictive impact of the SUB1154 amino acid substitutions identified using SIFT software, there is lack of evidence to conclude these substitutions are important or involved in binding to the host TIR domains. This would require in-depth analysis of the SUB1154 sequence to determine potential protein binding sites, for example, using MiST software (Hu *et al*., 2018). These sites could then be mutated and the ability for bovine TIR domains to bind to SUB1154 could be assessed. Alternatively, diazirine-based footprinting could be used to determine the specific Tcp binding site within SUB1154 through utilisation of photo-activated probes and MS (Manzi *et al*., 2016). Together, this would conclude the specific interaction occurring between the *S. uberis* SUB1154 protein and bovine TIR domain that results in priming of the NLRP3 inflammasome.

5.6. NLRP3 inflammasome activation signals

Production of IL-1β from BMMOs varied following stimulation with the different *S. uberis* strains isolated from the bovine mammary gland. No relationship was found between IL-1β production from BMMOs and the genetic distances or grouping of the *S. uberis* strains. Despite a moderate correlation between SUB1154 expression and IL-1β production, causality was not clearly defined. In addition, a reduction in IL-1β production was found from BMMOs stimulated with mutants independent of SUB1154 and BMMOs challenged with rSUB1154 alone, although transcribed *pro-IL-1β*, did not result in IL-1β production. This concluded that IL-1β production is SUB1154-dependent but the extent of IL-1β production is determined by the NLRP3 inflammasome activation signal. Therefore, further experimentation is needed to determine the NLRP3 inflammasome activation signal(s) following *S. uberis* stimulation. Activation signals can be provided from a variety of factors and may occur in combination. As *S. uberis* does not bind to TLR2 expressed on the extracellular surface of BMMOs and SUB1154 acts intracellularly, it is likely that the activation signal is due to the accumulation of molecules intracellularly.

5.6.1. Cathepsins

Phagocytosed compounds can be trafficked to the lysosome where they result in damage causing the release of cathepsins (Hornung *et al*., 2008). Cathepsins are lysosomal proteases

that become activated at low pH and aid in digestion of invading bacteria. There are 15 known cathepsins separated into three groups, serine proteases (A and G), aspartic proteases (D and E) and lysosomal cysteine proteases (B, C, F, H, K, L, O, S, V, X and W) (Turk *et al*., 2011). TLR3, TLR7/8 and TLR9 expressed on lysosomes recognise bacterial dsRNA, ssRNA and DNA CpG motifs respectively and activate cathepsins (Szulc-Dąbrowska *et al*., 2020). Gram positive bacteria interact with TLR2 on the extracellular membrane and TLR3 on intracellular membranes resulting in MyD88 dependent and independent signalling that leads to the production of pro-inflammatory cytokines TNF-α and IL-1β. These cytokines function as primary regulators of cathepsins L and S specifically and enhance their proteolytic activity (Creasy & McCoy, 2011). In macrophages, efficient phagocytosis and killing of *S. aureus* was found to be driven by cathepsin L (Müller *et al*., 2014); corresponding data for *S. uberis* has not been published.

To test this, expression of cathepsins could be detected in BMMOs challenged with *S. uberis* strain 0140J using qPCR. Initially, expression should be determined for cathepsin L and S and if this is unsuccessful then expression for the other cathepsins should be investigated. Once the presence of cathepsins are found, activity assays can be conducted on the respective cathepsins. Easily accessible assay kits can be purchased for cathepsins B, D, E, G, H, K, L and S. Cell lysates that contain the cathepsin of interest will cleave the synthetic substance added to release a fluorescent particle that can be measured using a spectrophotometer, with the amount of particle directly proportional to cathepsin activity (Abcam).

5.6.2. Ion fluxes

Ion fluxes of potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-) are markers of inflammasome activation. K^+ efflux is triggered by phagocytosis as when inhibited by cytochalasin B and latrunculin B in bone marrow-derived macrophages, K^+ efflux and NLRP3-dependent IL-1β secretion was impaired (Muñoz-Planillo *et al*., 2013). K^+ efflux alone was found to provide sufficient NLRP3 activation by facilitating the interaction between NLRP3 and NEK7, aiding inflammasome assembly (He *et al*., 2016). Extracellular K^+ levels could be quantified from the supernatant using a fluorometric assay following BMMO challenge with *S. uberis* strain 0140J (Abcam, ab252904) (Liu *et al*., 2021). Comparing the concentration of K^+ found in the supernatant of BMMOs in the presence and absence of *S. uberis* strain 0104J should be indicative of the use of these ions as an NLRP3 inflammasome activation signal. It is most likely that K^+ contributes to activation of the NLRP3 inflammasome, but it would need to be determined whether these ions act in conjunction with other ions.

NLRP3 can also be activated by the influx of Ca^{2+} from both intracellular and extracellular stores (Gong *et al*., 2018). Cellular stress may result in Ca^{2+} transportation from the endoplasmic reticulum (ER) into the cytoplasm via the inositol triphosphate receptor (IP_3R). Destabilisation of the lysosomal membrane may also result in an efflux of Ca^{2+} out of the lysosome and into the cytoplasm. Alternatively, extracellular Ca^{2+} can be transported into the cytoplasm by specific plasma membrane calcium ion channels, TRPM2, TRPM7 and TRPV2, or via the non-selective cation ATP-gated channel, P2X7R (Desai & Leitinger 2014; Gong *et al*., 2018; Wang *et al*., 2020; Pelegin, 2021). Ca^{2+} provides an NLRP3 activation signal by promoting NLRP3-ASC association. The excessive release of Ca^{2+} from the ER causes mitochondrial damage, inducing the production of ROS which further promotes NLRP3 activation (Gong *et al*., 2018).

To determine whether Ca^{2+} are involved in activating the NLRP3 inflammasome in BMMOs, the intracellular ion concentration should be compared between BMMOs in the presence and absence of *S. uberis* stimulation. Normal cytoplasmic Ca^{2+} concentration is approximately 100 nM (Gong *et al*., 2018) and can be measured using inductively coupled plasma optical emission spectrometry (ICP-OES), as described in Muñoz-Planillo *et al*., 2013. If Ca^{2+} is found to be an activation signal, then the source and method by which this occurs needs to also be determined. To determine whether the Ca^{2+} influx originates from extracellular stores, the plasma membrane receptors may be inhibited. The intracellular concentration of Ca^{2+} could be calculated following BMMO challenge with *S. uberis* in the presence and absence of plasma membrane receptor inhibitors. For example, A-438079 hydrochloride hydrate is a selective P2X7R antagonist that could be used to prevent Ca^{2+} influx from extracellular stores (Murakami *et al*., 2012; Fan *et al*., 2020; Pelegrin, 2021). As this receptor is non-selective, it would be interesting to also measure the concentration of K^+ in the supernatant following inhibition with this compound as it would indicate whether this receptor is used for such K^+ efflux. IP_3R inhibitors such as 2-APB (Torcis, 1224) and (-)-Xestospongin C (Torcis, 1280) could be used to prevent release of Ca^{2+} from the ER. Additionally, whether found to be Ca^{2+} dependent or independent, the production of ROS as an NLRP3 activation signal could also be measured using a fluorometric ROS assay (Abcam, ab186027).

Finally, Cl^- was found to be involved in NLRP3 activation. Following mitochondrial damage and the production of ROS, volume-regulated anion channels and chloride intracellular channels mediate Cl^- efflux out of the cell. This prevents cell swelling, promotes NEK7-NLRP3 interactions and induces ASC polymerisation to activate the NLRP3 inflammasome (Gong *et al*., 2018; Green *et al*., 2018). Similar to investigating the involvement of K^+ and Ca^{2+}, the extracellular concentration of Cl^- could be determined using a colorimetric assay (Sigma, MAK023).

Additionally, Cl^- channels could be inhibited using compounds such as 5-nitro-2-(3-phenylpropylamino) benzoic acid and 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) (Malekova *et al*., 2007). Comparison of the Cl^- concentration in the supernatant following BMMO challenge with *S. uberis* in the presence and absence of these inhibitors could be used to determine whether Cl^- efflux is an NLRP3 activation signal in the context of *S. uberis* stimulation.

Once the NLRP3 inflammasome activation signals are confirmed for *S. uberis* strain 0140J, it would be interesting to evaluate these signals for different *S. uberis* isolates. This may provide evidence for a trend between the activation signals and the production of IL-1β from stimulated BMMOs. Together, this would conclude how *S. uberis* primes and activates the BMMO NLRP3 inflammasome. Next would be how to target these priming and activation steps as a potential therapy for *S. uberis* causing bovine mastitis that would affect all strains.

5.7. Th17 cells and S. uberis intramammary infections

This project focused on the non-specific BMMO function in the initiation of an innate immune response to *S. uberis* following bacterial ingestion. However, BMMOs also contribute to the specific adaptive immune response through antigen processing and presentation to CD4+ T helper cells via MHCII (Denis *et al*., 2006). This interaction results in differentiation of the CD4+ T-cells into one of the five major Th subsets (Th1, Th2, Th17, Treg and Tfh). Each Th subset dictates a specific type of adaptive immunity which is dependent on the cause of the immune response. Th cells achieve this through promoting the cytotoxic CD8+ T-cell response and interacting with B cells to produce specific antibodies (Jeffery, 2023).

As with most infections, an increase in the T-cell population was observed 96h after *S. uberis* infection and 4 out of 6 cows were able to eliminate the infection without the need for therapeutic intervention (Tassi *et al*., 2013). However, antibiotics are usually administered at the onset of clinical signs of disease (48h after challenge in this case). Therefore, an effective vaccine needs to increase the T-cell population quickly before the onset of infection to avoid excessive mammary gland damage and the need for antibiotic therapy. Resolution of *S. uberis* infection coincided with the detection of IL-17A, suggesting the possible differentiation and development of the Th17 cell subset (Tassi *et al*., 2013). Intramammary challenge with ovalbumin resulted in detectable IL-17 in milk after only 14h (Rainard *et al*., 2013). This implies that a T-cell adaptive immune response could be generated and the infection cleared, eliminating progression to clinical signs. Therefore, further investigation needs to be conducted to determine *S. uberis* antigens that could be utilised to stimulate a fast Th17 cell

response. BMMOs play a crucial role in presenting antigens to T-cells resulting in their differentiation into Th17 cells, therefore this highlights the importance of the *S. uberis* SUB1154 protein as without it, initiation of the immune response by BMMOs is further delayed. Therefore, it may be important to incorporate this protein into a therapeutic to enhance the BMMO response specifically against *S. uberis.*

Conclusion

S. uberis is the predominant cause of bovine mastitis with no alternative to antibiotic therapy available to treat the disease. In order to develop an effective treatment, new targets must be identified and this can only happen through understanding the underlying pathogenesis and the bovine immune response to *S. uberis.* This study made significant progress in understanding the innate immune response to *S. uberis*, with the discovery that the SUB1154 protein provides the NLRP3 inflammasome priming signal in BMMOs, most likely via interactions with bovine TIR domains associated with TLR2. Further research needs to determine the NLRP3 inflammasome activation signal(s), and subsequently the involvement of BMMOs in establishment of the adaptive branch of the bovine immune response to *S. uberis*, with a focus on Th17 cells. Understanding the immune response to *S. uberis* will help in the discovery of potential stages of pathogenesis against which immunomodulatory therapeutics may be developed.

Supplementary Information

Table SI1. Cell isolation numbers and yield.

Whole milk (SCC/µL)	Whole milk (Total SCC)	Isolated cells (cells/µL)	Isolated cells (Total cell number)	Isolated cell yield (%)	BMMOs/well
36	$1.08x10^8$	880	$4.40x10^7$	40.7	55,911
36	$1.08x10^8$	892	$4.46x10^7$	41.3	68,842
51	$1.53x10^8$	810	$4.05x10^7$	26.5	51,363
116	$3.48x10^8$	790	$3.95x10^7$	11.4	65,233
90	$2.70x10^8$	600	$3.00x10^7$	11.1	48,487
100	$3.00x10^8$	740	$3.70x10^7$	12.3	59,203
73	$2.19x10^8$	1290	$6.45x10^7$	29.5	57,147
129	$3.87x10^8$	651	$3.26x10^7$	8.4	41,498
70	$2.10x10^8$	890	$4.45x10^7$	21.2	57,440
100	$3.00x10^8$	900	$4.50x10^7$	15.0	62,581
50	$1.50x10^8$	650	$3.25x10^7$	21.7	56,480
150	$4.50x10^8$	940	$4.70x10^7$	10.4	39,423
110	$3.30x10^8$	720	$3.60x10^7$	10.9	65,815
90	$2.70x10^8$	690	$3.45x10^7$	12.8	67,298
160	$4.80x10^8$	460	$2.30x10^7$	47.9	60,297
100	$3.00x10^8$	840	$4.20x10^7$	14.0	63,639
80	$2.40x10^8$	820	$4.10x10^7$	17.1	61,391
40	$1.20x10^8$	300	$1.50x10^7$	12.5	55,414
70	$2.10x10^8$	590	$2.95x10^7$	14.0	58,072
86	$2.58x10^8$	700	$3.50x10^7$	13.6	52,098
86	$2.58x10^8$	840	$4.20x10^7$	16.3	52,686
60	$1.80x10^8$	700	$3.50x10^7$	19.4	44,533
75	$2.25x10^8$	1100	$5.50x10^7$	24.4	60,277
80	$2.40x10^8$	600	$3.00x10^7$	12.5	68,741
150	$4.50x10^8$	660	$3.30x10^7$	7.3	60,050
50	$1.50x10^8$	725	$3.63x10^7$	24.2	55,557
60	$1.80x10^8$	540	$2.70x10^7$	15.0	62,276
45	$1.35x10^8$	650	$3.25x10^7$	24.1	44,736
35	$1.05x10^8$	400	$2.00x10^7$	19.0	44,093
48	$1.44x10^8$	630	$3.15x10^7$	21.9	42,615
40	$1.20x10^8$	555	$2.78x10^7$	23.1	56,035
70	$2.10x10^8$	690	$3.45x10^7$	16.4	62,141
97	$2.91x10^8$	1468	$7.34x10^7$	25.2	62,592
110	$3.30x10^8$	540	$2.70x10^7$	8.2	51,009
52	$1.56x10^8$	450	$2.25x10^7$	14.4	57,823
196	$5.88x10^8$	768	$3.84x10^7$	6.5	58,028
48	$1.44x10^8$	464	$2.32x10^7$	16.1	47,244
86	$2.58x10^8$	660	$3.00x10^7$	11.6	56,747
69	$2.07x10^8$	900	$4.50x10^7$	21.7	45,034
45	$1.38x10^8$	550	$2.75x10^7$	19.9	66,417
162	$4.86x10^8$	997	$4.99x10^7$	10.3	53,599
36	$1.08x10^8$	513	$2.57x10^7$	23.8	68,386
110	$3.30x10^8$	1000	$5.00x10^7$	15.2	68,328
55	$1.65x10^8$	575	$2.88x10^7$	17.5	50,612
70	$2.10x10^8$	540	$2.70x10^7$	12.9	58,891
40	$1.20x10^8$	600	$3.00x10^7$	25.0	44,652
57	$1.71x10^8$	578	$2.89x10^7$	16.9	41,620
135	$4.05x10^8$	850	$4.25x10^7$	10.5	61,495

64	$1.92x10^8$	650	$3.25x10^7$	16.9	46,471
76	$2.28x10^8$	800	$4.00x10^7$	17.5	53,347
140	$4.20x10^8$	640	$3.20x10^7$	7.6	56,145
80	$2.40x10^8$	900	$4.50x10^7$	18.8	49,120
140	$4.20x10^8$	800	$4.00x10^7$	9.5	60,043
44	$1.32x10^8$	468	$2.34x10^7$	17.7	46,414
42	$1.26x10^8$	694	$3.47x10^7$	27.5	45,267
58	$1.74x10^8$	688	$3.44x10^7$	19.8	50,174
64	$1.92x10^8$	1360	$6.80x10^7$	35.4	40,135
36	$1.08x10^8$	611	$3.06x10^7$	28.3	46,867
36	$1.08x10^8$	487	$2.44x10^7$	22.6	41,635
141	$4.23x10^8$	1000	$5.00x10^7$	11.8	59,209
140	$4.20x10^8$	1059	$5.30x10^7$	12.6	63,322
72	$2.16x10^8$	950	$4.75x10^7$	22.0	50,520
23	$6.90x10^7$	430	$2.15x10^7$	31.2	40,670
57	$1.71x10^8$	1200	$6.00x10^7$	35.1	51,985
40	$1.20x10^8$	450	$2.25x10^7$	18.8	52,929
63	$1.89x10^8$	783	$3.92x10^7$	20.7	64,172
50	$1.50x10^8$	422	$2.11x10^7$	14.1	40,060
65	$1.95x10^8$	388	$1.94x10^7$	9.9	44,172
53	$1.59x10^8$	849	$4.25x10^7$	26.7	56,440
48	$1.44x10^8$	539	$2.70x10^7$	18.8	50,230
58	$1.74x10^8$	342	$1.71x10^7$	9.8	48,823
78	$2.34x10^8$	577	$2.89x10^7$	12.4	53,850
110	$3.30x10^8$	1125	$5.63x10^7$	17.1	60,686
121	$3.63x10^8$	1305	$6.53x10^7$	18.0	62,421
50	$1.50x10^8$	565	$2.83x10^7$	18.9	50,022
84	$2.52x10^8$	650	$3.25x10^7$	12.9	56,179
110	$3.30x10^8$	950	$4.75x10^7$	14.4	58,217
65	$1.95x10^8$	785	$3.93x10^7$	20.2	52,390
47	$1.41x10^8$	679	$3.40x10^7$	24.1	47,645
44	$1.32x10^8$	426	$2.13x10^7$	16.1	47,737
90	$2.70x10^8$	1044	$5.22x10^7$	19.3	61,568
123	$3.69x10^8$	1422	$7.11x10^7$	19.3	58,175
125	$3.75x10^8$	1687	$8.44x10^7$	22.5	59,399
68	$2.04x10^8$	964	$4.82x10^7$	23.6	52,275
83	$2.49x10^8$	750	$3.75x10^7$	15.1	50,655
67	$2.01x10^8$	852	$4.26x10^7$	21.2	46,326
65	$1.95x10^8$	741	$3.71x10^7$	19.0	46,905
65	$1.95x10^8$	763	$3.82x10^7$	19.6	53,143
60	$1.80x10^8$	656	$3.28x10^7$	18.2	49,464
50	$1.50x10^8$	1030	$5.15x10^7$	34.3	65,534
65	$1.95x10^8$	728	$3.64x10^7$	18.7	53,989
54	$1.62x10^8$	518	$2.59x10^7$	16.0	40,814
70	$2.10x10^8$	1600	$8.00x10^7$	38.1	67,455
28	$8.40x10^7$	617	$3.09x10^7$	36.8	47,144
43	$1.29x10^8$	579	$2.90x10^7$	22.5	44,556
61	$1.83x10^8$	516	$2.58x10^7$	14.1	49,133
76	**$2.29x10^8$**	**758**	**$3.79x10^7$**	**19.1**	**54,041**

For each 3L of milk collected, the whole milk somatic cell count (SCC/µL) was measured using a DeLaval cell counter and total cell number calculated. During the cell isolation protocol, the cells were resuspended in 50 mL PBS and cells/µL were measured and total cell number calculated. The yield was calculated for the percentage of isolated cells obtained from whole milk. Following washing of cells to remove contaminating leukocytes, from the three wells set aside, bovine mammary macrophages (BMMOs) were removed and the number of BMMOs/well was calculated using a haemocytometer. The mean for each column is presented in the bottom row.

Table SI2. SUB1154 amino acid changes between *S. uberis* strains.

SUB1154	Amino Acid Position																		
S. uberis strain	1	7	78	79	80	96	118	157	167	216	219	229	291	336	379	474	558	561	612
0140J	M	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	A	L
SUD63	-	H	S	K	S	A	K	L	Q	S	G	V	A	V	V	A	A	A	I
SUD67	I	D	P	K	S	A	N	L	E	S	G	I	A	A	V	A	A	G	L
SUD69	I	H	S	K	S	A	K	L	Q	S	G	V	A	V	V	A	A	A	I
SUD75	I	N	P	K	S	A	K	L	E	N	G	V	A	A	V	A	G	A	L
SUD76	-	N	P	K	S	A	K	L	E	N	G	V	A	A	V	A	G	A	L
SUD79	I	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	A	L
SUD84	-	N	P	N	N	T	N	I	E	S	S	V	S	A	F	S	A	A	L
SUD221	-	D	P	K	S	A	N	L	E	S	G	I	A	A	V	A	A	G	L
SUD248	-	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	G	L
SUD249	I	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	G	L
SUD250	-	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	G	L
SUD276	I	D	P	K	S	A	N	L	E	S	G	I	A	A	V	A	A	G	L
SUD277	-	H	S	K	S	A	K	L	Q	S	G	V	A	V	V	A	A	A	I
SUD285	I	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	G	L
SUD287	-	D	P	K	S	A	N	L	E	S	G	I	A	A	V	A	A	A	L
SUD511	-	H	S	K	S	A	K	L	Q	S	G	V	A	V	V	A	A	A	I
SUD514	-	H	S	K	S	A	K	L	E	S	G	I	A	A	V	A	A	A	L

Amino Acid Position																		
632	641	664	755	822	955	1012	1018	1027	1030	1037	1056	1077	1080	1084	1090	1100	1109	1122
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	Y	S	F
R	N	P	G	R	I	N	H	S	S	Y	G	F	R	E	A	R	S	L
R	N	P	G	R	T	S	H	R	S	Q	G	S	S	K	A	H	S	F
R	N	P	G	R	I	N	H	S	S	Y	G	F	R	E	A	R	S	L
S	K	P	G	R	T	S	H	R	Y	H	G	S	S	K	A	H	S	F
S	K	P	G	R	T	S	H	R	Y	H	G	S	S	K	A	H	S	F
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	Y	S	F
S	K	S	V	R	I	N	N	S	S	H	G	S	S	K	T	H	S	F
R	N	P	G	R	T	S	H	R	S	Q	G	S	S	K	A	H	S	F
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	H	S	F
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	H	S	F
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	H	S	F
R	N	P	G	R	T	S	H	R	S	Q	G	S	S	K	A	H	S	F
R	N	P	G	R	I	N	H	S	S	Y	G	F	R	E	A	R	S	L
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	H	S	F
R	K	P	G	K	T	S	H	R	Y	H	D	S	S	E	A	R	C	L
R	N	P	G	R	I	N	H	S	S	Y	G	F	R	E	A	R	S	L
R	N	P	G	R	T	N	H	R	S	H	G	S	S	K	A	H	S	F

The SUB1154 amino acid sequences from the *S. uberis* strains isolated from the bovine mammary gland were aligned to the *S. uberis* strain 0140J SUB1154 sequence using MEGA 11. The strains that have a different amino acid compared to the *S. uberis* strain 0140J are highlighted in purple with the amino acid position referenced at the top.

Table SI3. SUB0144 amino acid changes between *S. uberis* strains.

SUB0144	Amino Acid Position																		
S. uberis strain	20	29	58	70	101	108	138	151	167	168	187	189	229	281	305	310	350	365	428
0140J	V	C	V	V	K	E	V	H	N	Y	M	A	C	R	N	P	N	L	Y
SUD63	V	C	V	V	K	E	V	H	Y	F	V	T	Y	R	N	V	K	S	Y
SUD67	I	C	V	V	N	E	M	H	N	Y	V	A	Y	Q	N	P	K	L	Y
SUD69	I	C	V	V	N	E	M	H	N	Y	V	A	Y	Q	N	P	K	L	Y
SUD75	V	C	V	V	K	E	V	H	Y	F	V	T	Y	R	E	V	K	S	Y
SUD76	V	C	V	V	K	E	V	H	Y	F	V	T	Y	R	E	V	K	S	Y
SUD79	V	C	V	V	N	K	M	H	N	Y	V	A	C	Q	N	P	K	L	Y
SUD84	V	S	V	V	K	E	V	H	Y	Y	V	T	C	R	N	V	K	S	Y
SUD221	V	S	V	I	N	E	M	H	N	Y	V	A	Y	R	N	P	K	S	Y
SUD248	V	S	V	V	N	E	M	R	N	Y	V	A	Y	R	N	P	K	S	Y
SUD249	V	S	V	V	N	E	M	R	N	Y	V	A	Y	R	N	P	K	S	Y
SUD250	V	S	V	I	N	E	M	H	N	Y	V	A	Y	R	N	P	K	S	Y
SUD276	V	S	V	V	N	E	M	R	N	Y	V	A	Y	R	N	P	K	S	Y
SUD277	V	C	I	V	K	E	V	H	Y	Y	V	T	Y	Q	N	P	K	L	Y
SUD285	V	S	V	V	N	E	M	R	N	Y	V	A	Y	R	N	P	K	S	Y
SUD287	V	S	V	V	K	E	V	H	Y	F	V	T	C	R	E	V	K	S	Y
SUD511	V	C	I	V	K	E	V	H	Y	Y	V	T	Y	Q	N	P	K	L	Y
SUD514	I	C	V	V	N	E	M	Q	N	Y	V	A	Y	Q	N	P	K	S	C

The SUB0144 amino acid sequences from the *S. uberis* strains isolated from the bovine mammary gland were aligned to the *S. uberis* strain 0140J SUB1154 sequence using MEGA 11. The strains that have a different amino acid compared to the *S. uberis* strain 0140J are highlighted in purple with the amino acid position referenced at the top.

Table SI4. SUB0881 amino acid changes between *S. uberis* strains.

SUB0881	Amino Acid Position		
***S. uberis* strain**	**28**	**50**	**67**
0140J	I	R	A
SUD63	V	K	A
SUD67	V	K	A
SUD69	I	R	A
SUD75	-	-	-
SUD76	-	-	-
SUD79	I	K	A
SUD84	-	-	-
SUD221	I	R	A
SUD248	I	K	A
SUD249	I	K	A
SUD250	I	K	A
SUD276	I	R	A
SUD277	I	K	V
SUD285	I	K	A
SUD287	-	-	-
SUD511	I	K	V
SUD514	I	K	V

The SUB0881 amino acid sequences from the *S. uberis* strains isolated from the bovine mammary gland were aligned to the *S. uberis* strain 0140J SUB1154 sequence using MEGA 11. The strains that have a different amino acid compared to the *S. uberis* strain 0140J are highlighted in purple with the amino acid position referenced at the top.

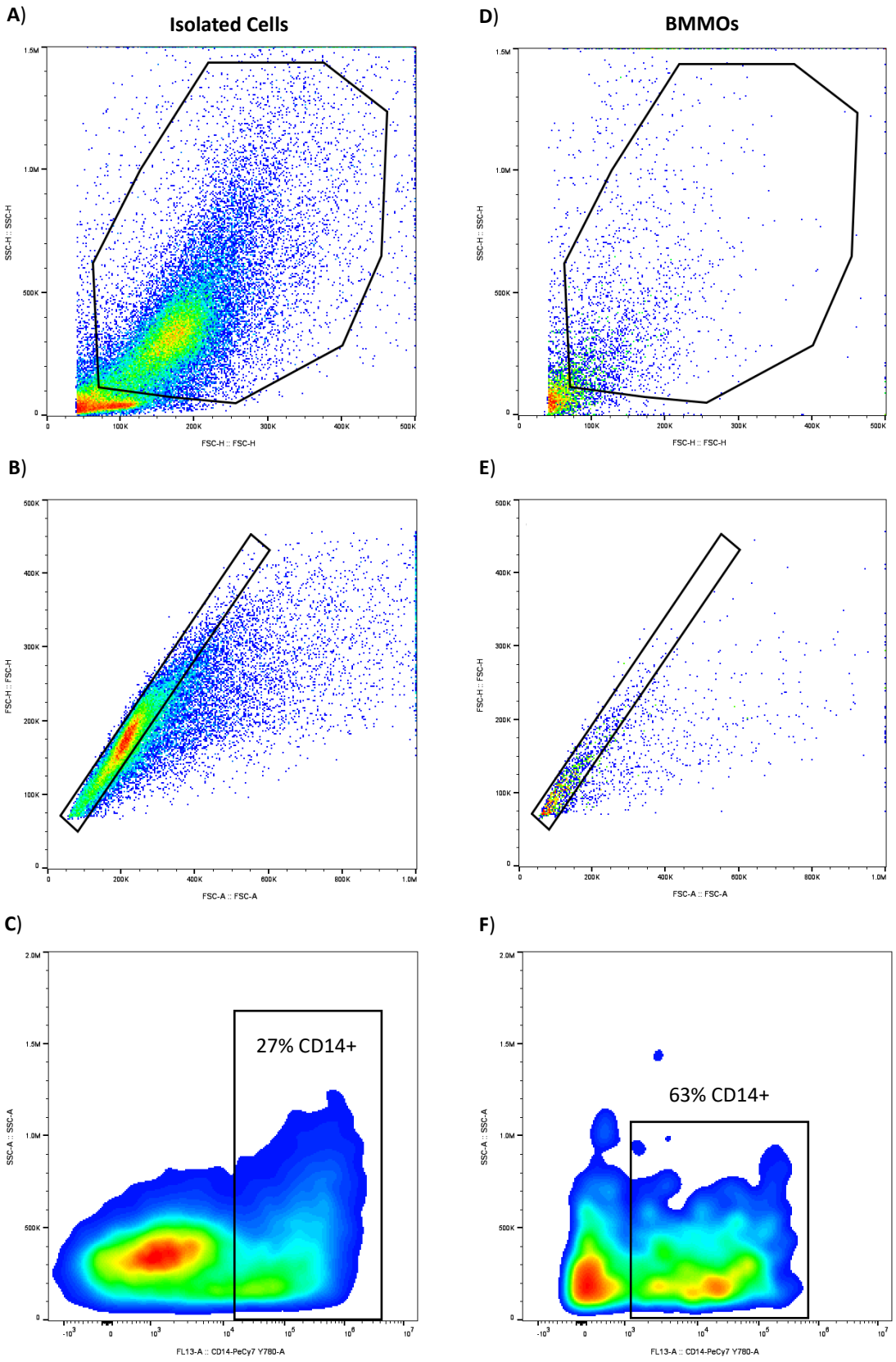
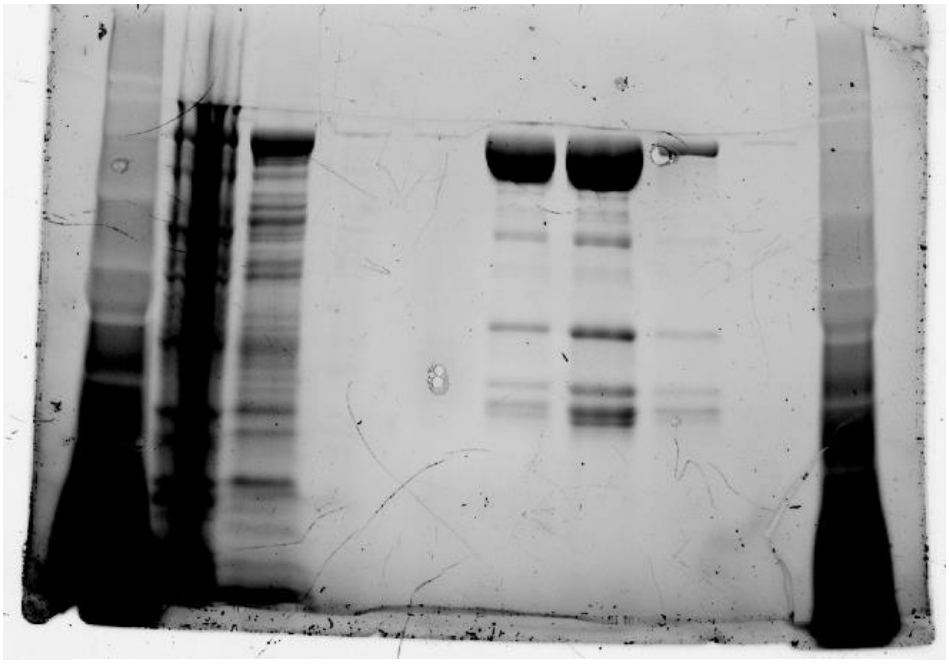


Fig SI1. Gating to determine percentage of CD14+ cells. Cells were stained with CD14 PE-Cy7 and flow cytometry was used to determine the percentage of CD14+ cells in the somatic cell population (**A-C**) and the isolated BMMO population (**D-F**). All cells, excluding likely debris were gated (**A** and **D**), followed by identification of the single cell population (**B** and **E**). Finally, CD14+ cells were selected (**C** and **F**) and the percentage calculated (Fig 2.2).

A)



B)

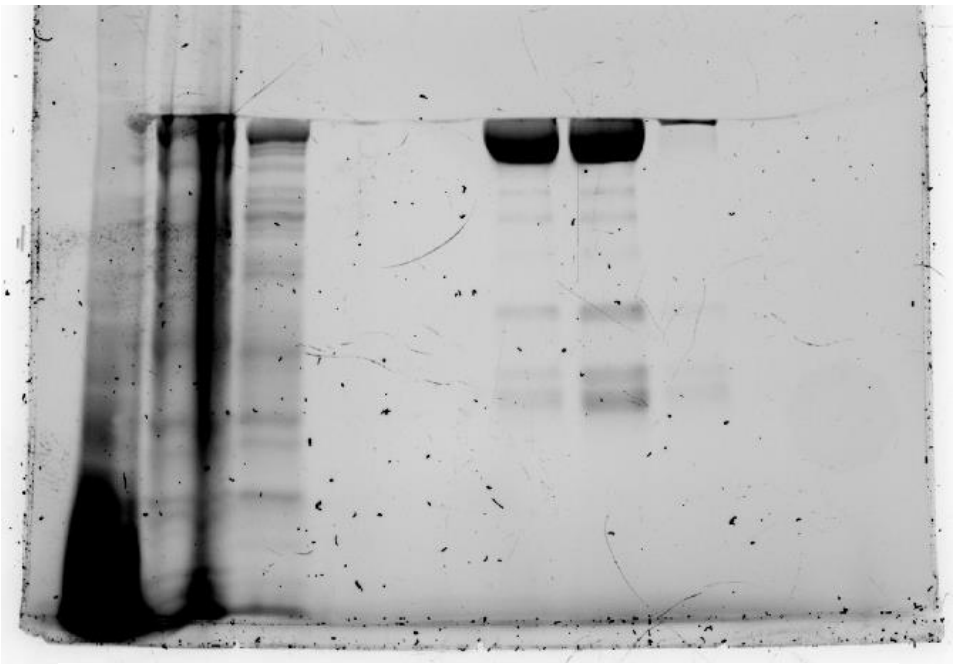


Fig SI2. Eluted fractions of the purified recombinant SUB1154 proteins. Recombinant SUB1154 (**A**) and rSUB1154NP (proteolytically compromised) (**B**) proteins were purified from *E. coli* host using the pQE-1 expression plasmid containing an ampicillin resistance marker gene, T5 promoter and a LacO operon. HisPur™ Ni-NTA Chromatography protein purification cartridges eluted the purified proteins and SDS-PAGE was used to determine which flow-through fractions contained the rSUB1154/NP proteins. L = protein ladder (11-250 kDa); CE = cell extract; SP = soluble proteins; F = fraction. The gels show that rSUB1154/NP were within fractions 3 and 4.

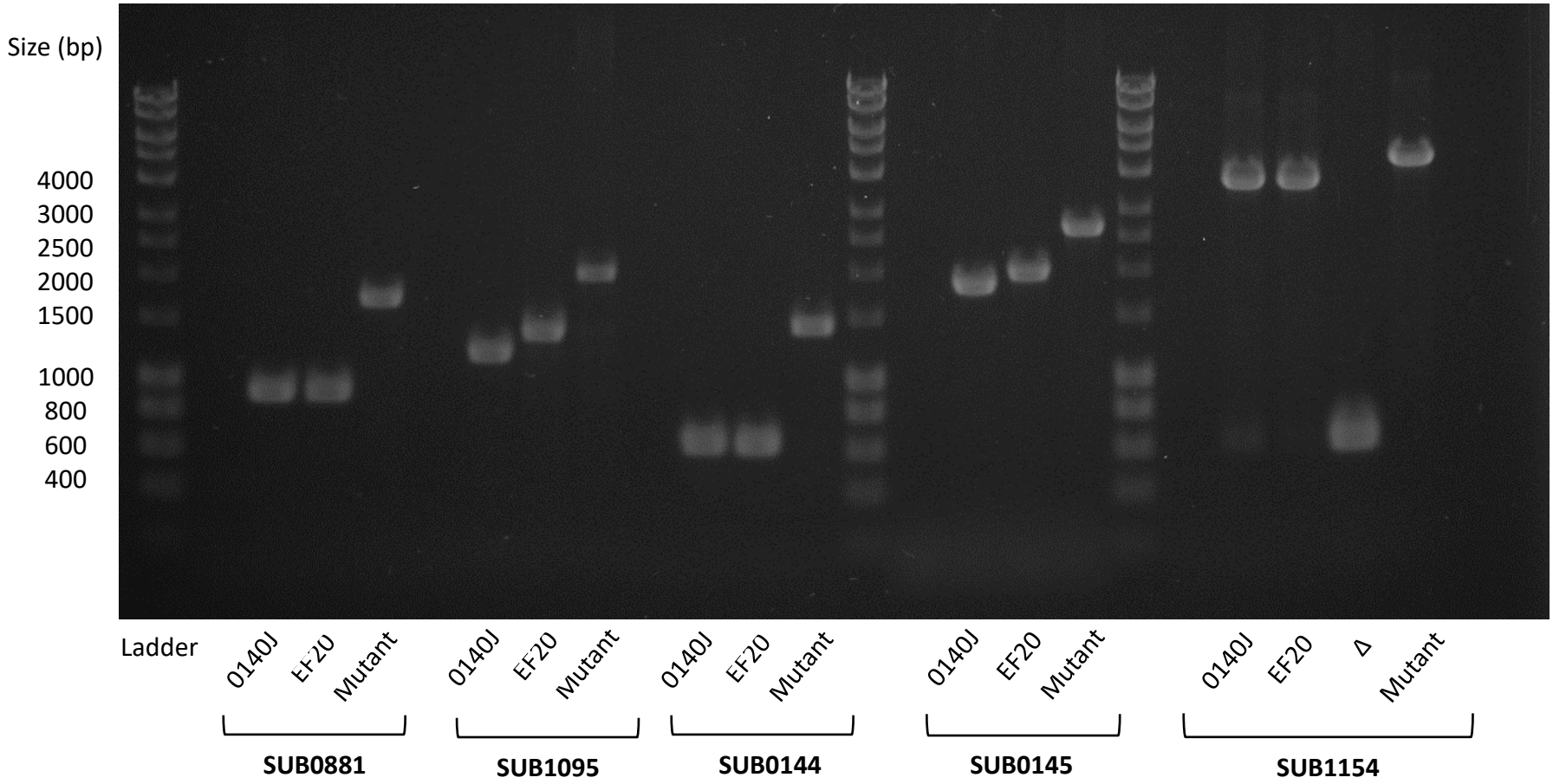


Fig SI3. *S. uberis* mutant strains confirmation. DNA was extracted from *S. uberis* strains 0140J, EF20, SUB1154 deletion (0140JΔ*sub1154*) and the insertional mutant strains 0140J::ISS1::*sub0881*, 0140J::ISS1::*1095*, 0140J::ISS1::*0144*, 0140J::ISS1::*0145* and 0140J::ISS1::*sub1154* cultures, amplified using five sets of PCR primers, one for each of the mutant gene targets (Table 4.1) and imaged through gel electrophoresis. Base pairs (bp).

Professional internship reflective statement

As part of my PhD, I undertook a three-month placement at Porterhouse Medical in Nottingham. I experienced what it would be like to work in a medical communications agency as an associate medical writer. I completed my placement here as I have always been intrigued as to what a medical writer would do but found it hard to get a clear answer on the day-to-day details when conducting my own research. Therefore, I thought this would be a great opportunity to uncover this and determine whether I would be interested in a career path within medical communications.

Porterhouse Medical is a growing company which has its headquarters in Reading, with several other small offices around the UK and the USA. Across the company, teams are associated with a few different clients, mostly consisting of pharmaceutical companies. I was part of the Bayer and Takeda group which focused on ophthalmology and epilepsy. Each team consisted of medical writers and client services at a range of different levels, who all came from a variety of different backgrounds. Most of the medical writers had completed a PhD, with some of the client service staff having no scientific background.

The Nottingham office adopted a hybrid working week with the majority of time spent working from home with every Thursday as an office day. I enjoyed the work from home element as all the meetings were on teams anyway due to work being conducted across offices. However, I valued the one day a week in the office as the Nottingham team were extremely friendly and it was fun to spend the day together. This team had regular social events during lunch and after work that I partook in.

The majority of work I was assigned consisted of making and editing PowerPoint presentations and reference linking statements to research papers. These presentations ranged from training slides for new starters at pharmaceutical companies, updates on clinical trial data and presentations that would be presented at international conferences by healthcare professionals. It was interesting to see how strict and precise the house-style was with specific hyphens, grammar and punctuation used at specific points. Although tedious, I did quite enjoy lining everything up on the slides so there was no movement between slide transitions, and this has definitely improved my PowerPoint skills. The activity I most enjoyed was watching presentations at conferences and then making summary materials that were relevant to the

client. As a more creative task, I was involved in helping make a company newsletter and audio editing and transcribing a podcast, this I did not enjoy.

I had the opportunity to experience the whole pipeline of how a piece of work is generated by writing an article for their website. To coincide with the development of therapeutics for epilepsy by one of the clients I was involved with, I wrote and published an article on the Porterhouse Medical website on Dravet syndrome. By completing this I got to experience the senior review and editorial processes, as well as communicating with the creative team to make a website banner.

Overall, I enjoyed my placement and was glad to experience what it would be like to work as a medical writer. Although the corporate environment showed to have lots of benefits, including free private healthcare, many social events and lots of meetings that took up a lot of time, I think this career is not for me. I really missed being in the lab and working independently. Despite having to learn a broad range of disease areas, I enjoy being challenged, learning in-depth about immunology specifically and problem solving. These are all areas I experienced during my PhD. Therefore, I believe that I would much prefer a career as an academic researcher than in the medical communications industry.

References

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